IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant	:	Timothy J. O'Leary et al.	
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Examiner	:	CALAMITA, H.	
Docket No.	:	AFIP 03-16 01	
Customer No.	:	27370	

For: IMMUNOLIPOSOME-NUCLEIC ACID AMPLIFICATION (ILNAA) ASSAY

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. BOX 1450
Alexandria, VA 22313-1450

Sir:

In response to the final Office Action of June 18, 2008, and further to the Notice of Appeal filed on November 13, 2008, Applicants submit the following Appeal Brief pursuant to 37 CFR 41.37.

Real Party in Interest

The real party in interest is the United States Government as represented by the Secretary of the Army. An assignment has not yet been filed for this application.

Related Appeals and Interferences

There are no related appeals, interferences, or judicial proceedings known to Applicants, Applicants' representative, or the United States Government as represented by the Secretary of the Army.

Status of Claims

Claims 16-18, 20-24, 26-31, 33, 35, 37-38, and 41-46 stand finally rejected and are appealed.

Claims 32, 34, and 39 are objected to as being dependent on a rejected base claim and are thus not appealed.

Claims 1-15, 19, 25, 36, and 40 are canceled. No claim is allowed.

Status of Amendments

In response to the final Office Action dated June 18, 2008, an Amendment After Final Rejection with claim amendments was filed on September 3, 2008. Pending dependent Claim 19 was canceled and its subject matter was incorporated into independent Claim 16. Claim 43 was amended to correct typographical errors.

A first Advisory Action was mailed on September 18, 2008 incorrectly stating that the Amendment After Final Rejection would not be entered because it raised new issues and new matter. However, when it was brought to the Examiner's attention that the Amendment simply incorporated the subject matter of dependent Claim 19 into independent Claim 16, she mailed a second Advisory Action on October 1, 2008 indicating that the proposed claim amendments would be entered for purposes of an Appeal.

Summary of Claimed Subject Matter

1. Independent Claim 16

The present invention is directed to a method for detecting an analyte with an immunoliposome-nucleic acid amplification (ILNAA) assay comprising (1) encapsulating 50 to 1,000 identical nucleic acid segments within closed shell liposomal bilayers, (2) associating receptors to the extravesicular surface of the liposomal bilayers, (3) exposing the receptors to an immobilized target analyte, which binds to the liposomal bilayer associated receptors; (4) removing unbound liposomal bilayers; (5) lysing the bound liposomal bilayers to release the nucleic acid segments; (6) amplifying the

nucleic acid segments released from the liposomal bilayers, and (7) detecting the amplification products of the nucleic acid segments to quantify the amount of the target analyte, as recited in independent Claim 16. *See* paragraph [0016] on page 8, lines 1-22 of the specification; paragraph [0028] on page 11, line 6 – page 12, line 2 of the specification; and paragraphs [0033]-[0034] on page 13, line 5 – page 14, line 5 of the specification.

FIGS. 10-11 illustrate an immunoliposome with encapsulated nucleic acid segments having a receptor (antibody) associated to the extravesicular surface via a spacer arm. In FIG. 10, the liposome is bound to an antigen (biotoxin – target analyte) on a substrate, and in FIG. 11 an antibody complex is bound to the immunoliposome and the substrate:

FIG. 10

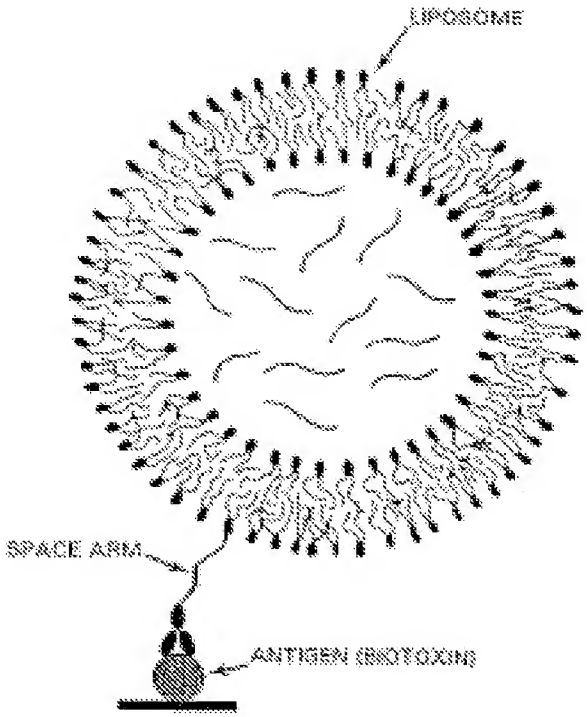
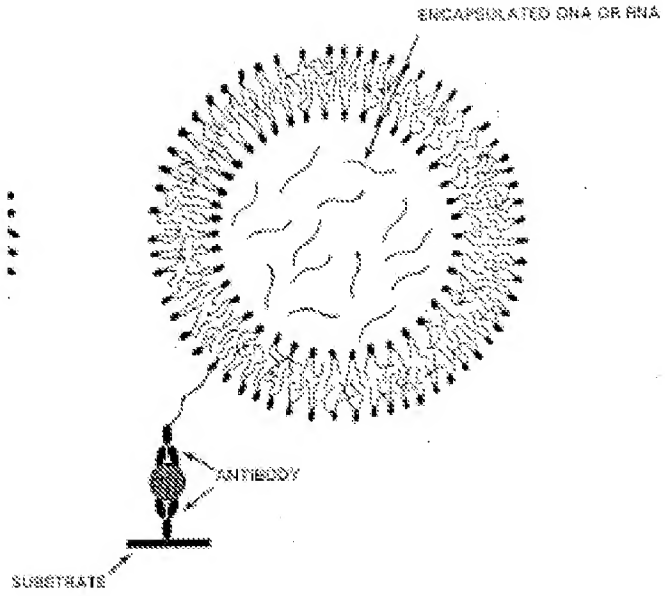


FIG. 11



Accordingly, this invention relates to a new assay system and method for detecting extremely small quantities (as few as 10-1000 molecules) of compounds for which specific receptors, such as antibodies, exist. *See* paragraph [0001] on page 1, lines 1-4 of the specification and paragraph [0016] on page 8, lines 1-8 of the specification.

2. Independent Claim 43

The present invention is also directed to a method for detecting an analyte with an immunoliposome-nucleic acid amplification assay comprising: (1) encapsulating a plurality of identical nucleic acid segments within closed shell liposomal bilayers, (2) incorporating receptors into the outer surface of said liposomal bilayers; and (3) exposing the receptors to a target analyte, causing aggregation of the receptors within the plane of the liposomal bilayers, wherein the aggregation causes the liposomal bilayers to become unstable leading to spontaneous rupture of the liposomal bilayers, and release of the nucleic acid segments. *See* paragraph [00209] on page 58, line 20 –

page 59, line 8 of the specification. The benefit of this assay is that no immobile phase is required.

Grounds of Rejection to be Reviewed Upon Appeal

1. Claims 16-17, 21-28, 31, 33, 35-38, 40-41 and 43 [***sic:*** Claim 25 was canceled and Claim 31 is the subject of a different rejection below] were rejected under 35 U.S.C. 103(a) over Singh et al. (Anal. Chem., 2000) in view of Wu et al. (Letters in Applied Microbiology, 2001).

2. Claim 18 was rejected under 35 U.S.C. 103(a) over Singh et al. in view of Wu et al. and further in view of Cao et al. (The Lancet, 2000).

3. Claims 20, 42, and 46 were rejected under 35 U.S.C. 103(a) over Singh et al. in view of Wu et al. and further in view of U.S. Patent No. 4,704,355 (Bernstein).

4. Claims 29-30 were rejected under 35 U.S.C. 103(a) over Singh et al. in view of Wu et al. and further in view of U.S. Patent No. 6,503,452 B1 (Boxer et al.).

5. Claim 31 was rejected under 35 U.S.C. 103(a) over Singh et al. in view of Wu et al. and further in view of Huang et al. (Biotechniques, 1996).

6. Claims 44-45 were rejected under 35 U.S.C. 103(a) over Singh et al. in view of Wu et al. and further in view of Bailey et al. (Biochimica et Biophysica Acta, 2000).[1]

[1] As noted, Claims 32, 34, and 39 are objected to as being dependent on a rejected base claim and are thus not appealed.

Argument

I. The Rejection of Claims 16-17, 21-28, 31, 33, 35-38, 40-41 and 43 over Singh et al. in view of Wu et al.

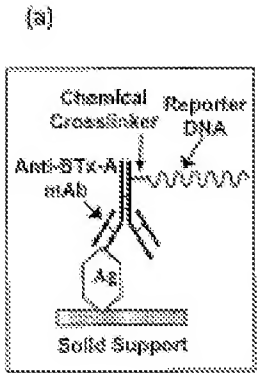
A. Claims 16-17, 24, 26-28, 33, 35-37, and 40-41

1. The Combination of Singh et al. and Wu et al. Does Not Teach the Claimed Invention

Singh et al. discloses using liposomes containing gangliosides as probes for detecting bacterial toxins. To impart signal generation capability to the liposomes, fluorophore-labeled lipids (rhodamine-labeled lipid) are incorporated into the bilayer of the liposomes. The fluorescent liposomes were used in sandwich fluoroimmunoassays for tetanus, botulinum, and cholera toxins and as low as 1 nM of each toxin could be detected (page 6019, left hand column). The signal molecules (i.e., rhodamine-labeled lipids) may be encapsulated, although "leakage upon storage is a serious concern" (page 6020, right hand column, emphasis added).

As acknowledged by the Examiner, Singh et al. does not teach or suggest encapsulating a plurality of identical nucleic acid segments within closed shell liposomal bilayers; amplifying the nucleic acid segments released from the liposomal bilayers, and detecting the amplification products of the nucleic acid segments to quantify the amount of the target analyte (final Office Action at page 5).

Wu et al. does not overcome the deficiencies of Singh et al. Wu et al. discloses using immuno-PCR to develop a sensitive assay to detect botulinum neurotoxin type A antigen (page 32). DNA-antibody conjugates are formed by the direct covalent attachment of a DNA amplification substrate to an antibody of interest. Wu et al. explicitly teach and require reporter DNA covalently linked to antibodies through amine and sulphhydryl groups (page 32, right hand column). *See* FIG. 1(a) on page 323:



This assay achieves a detection limit of roughly 6,000,000 molecules. *See* instant specification discussing Wu et al. at paragraph [0012], page 6, lines 7-14.

There is no teaching or suggestion to substitute 50-1,000 identical nucleic acid segments without covalently linked antibodies for the rhodamine-labeled lipids of Singh et al. In particular, there is no teaching in either of Singh et al. or Wu et al. to decouple DNA nucleic acid segments from an antibody and to encapsulate them individually in liposomes, much less so in view of Singh et al.'s teaching to incorporate signal lipids into the liposome bilayer and that encapsulation within the liposome raises serious leakage concerns upon storage. At the June 12, 2007 interview, the Examiner indicated that there were references other than Wu et al. which teach the use of DNA markers by themselves without covalent attachment to antibodies. No such references have ever been applied against the claimed invention.

Thus, the combined teachings of Singh et al. and Wu et al. do not teach or suggest encapsulating 50 to 1,000 identical nucleic acid segments within closed shell liposomal bilayers and associating receptors to the extravesicular surface of the liposomal bilayers, as recited in independent Claim 16.

2. Any *Prima Facie* Case of Obviousness Has Been Rebutted

The Examiner asserts that it would have been *prima facie* obvious to one of ordinary skill in the art to use the liposomes of Singh et al. with nucleic acid reports as taught by Wu et al. to detect the presence of an analyte with greater sensitivity. The Examiner asserts that the DNA reporters disclosed by Wu et al. enable detection of analytes at low levels because DNA markers improve sensitivity from 100 fold to 1000 fold over standard immunoassay methods (final Office Action at page 8).

However, the 100 to 1000 fold improvement disclosed in Wu et al. is the result of comparing immuno-PCR assays using DNA-antibody conjugates to ELISA assays in which an enzyme is conjugated to an antibody. Again, there is absolutely no teaching of using DNA sequences as markers by themselves, apart from being linked to antibodies or enzymes, in any kind of assay.

The Examiner's rationale is that there is a simple substitution of one known element for another to obtain predictable results or that combining prior art elements according to known methods would yield predictable results. Aside from being an improper combination of art as discussed above, Applicants have rebutted this rationale by demonstrating that:

(a) one of ordinary skill in the art could not have encapsulated nucleic acid segments within the liposomes of Singh et al.;

(b) the claimed invention achieves superior and unpredicted detection results; and

(c) secondary considerations have established long felt but unsolved needs and failure of others.

a. The Combination of Singh et al. and Wu et al. Would Render An Inoperable Method

As noted in the in the Combined Declaration Under 37 C.F.R. 1.131 and 1.132 filed on February 12, 2008 (131/132 Declaration[2]), the method of liposome formation in Singh et al. is only capable of encapsulating about 4 amplicons per liposome, which is unacceptable for the claimed method for detecting an analyte with an immunoliposome-nucleic acid amplification assay. Contrary to the Examiner's reasoning, one of ordinary skill in the art could not simply "substitute DNA markers [of Wu et al.] for the Rhodamine marker of Singh…" (Office Action dated October 12, 2007 at page 10).

It is axiomatic that if a proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *See* MPEP 2143.01. In view of the 131/132 Declaration, Applicants have established that trying to incorporate nucleic acid segments into the liposomes of Singh et al. would render an inoperable method for detecting an analyte with an immunoliposome-nucleic acid amplification (ILNAA) assay.

In the second Advisory Action, the Examiner for the first time dismisses the discussion in the 132 Declaration as "hypothetical" and that one of ordinary skill in the art would know to increase the concentrations of nucleic acid segments in the solution in which liposomes are formed (Second Advisory Action at page 2).

First, the extrusion method of Singh et al. would at best only allow for passive encapsulation of nucleic acid segments in liposomes. *See* numbered paragraph 16 of the 131/132 Declaration. Based upon the diameter of the liposomes (see Table 2 of Singh et al.) and lipid mixture of Singh et al. (see Experimental section of Singh et al.), the result is clear that Singh et al. could not encapsulate the 50-1,000 identical nucleic acid segments as claimed. *See* numbered paragraphs 17-19 of the 131/132

[2] The 131/132 Declaration is cited in the attached Evidence Appendix and a copy is attached hereto. As only the 132 section of the 131/132 Declaration is relied upon in this Appeal, the 131 Section is not discussed herein and those exhibits are not included.

Declaration. Accordingly, simply adding more nucleic acid segments to a solution as asserted by the Examiner would not result in a positive encapsulation of a greater number of nucleic acid segments within a specific spatial region.

Second, the discussion and calculations in the 132 Declaration are based on sound scientific reasoning. The Declaration evidence is entitled to fair weight and the Examiner has erred by characterizing the analysis as "merely hypothetical." *See* Ex Parte Tanksley et al., Board of Patent Appeals and Interferences, 37 U.S.P.Q.2d 1382 (1994), in which the Board noted that the opinion in a Declaration was based on a detailed statistical analysis involving logic and sound scientific reasoning, along with literature references. The Board in Ex Parte Tanskley et al. stated that the declaration evidence was entitled to fair weight and that examiner erred by (1) characterizing the analysis as "merely hypothetical," and (2) summarily dismissing the declaration as containing "unsupported opinions."

b. The Claimed Invention Achieves Superior Detection Results

In addition, the results of the claimed invention could in no way have been predicted. As discussed in the 131/132 Declaration, the claimed ILNAA assay achieves a detection limit of 10-1000 molecules of toxin in subattomolar quantities (i.e., below 1 x 10^{-18} moles). In fact, the immunoassay method can achieve a detection limit of 10 molecules of cholera toxin (17 x 10^{-24} moles or 17 yoctomoles) and 12 molecules of botulinum neurotoxin type A (20 yoctomoles).

In contrast, the method of Singh et al. teaches an assay method with a detection limit of 1 x 10^{-10} moles or 0.1 nanomoles for botulinum neurotoxin (page 6024). The method of Wu et al. teaches an assay with a detection limit of 33 x 10^{-18} moles or 18 attomoles for botulinum neurotoxin type A (Abstract).

As shown in the Table of the 131/132 Declaration, the ILNAA assay of the present invention is 5 x 10^{12} times more sensitive than the method of Singh et al. and 2

x 10^6 times more sensitive than the method of Wu et al. *See* Table on page 6 of the 131/132 Declaration.

Further, even if there were a 100 to 1000 fold improvement in the results of Singh et al., as asserted by the Examiner, this would at best lead to a detection level of 1 x 10^{-13} moles (an 1,000 fold improvement of 1 X 10^{-10} moles). However, the claimed ILNAA assay would still be 5 Billion times more sensitive! *See* Table on page 6 of the 131/132 Declaration.

Accordingly, in view of the 131/132 Declaration, Applicants have rebutted the Examiner's *prima facie* case of obviousness. As stated in MPEP 2145:

> If a *prima facie* case of obviousness is established, the burden shifts to the applicant to come forward with arguments and/or evidence to rebut the *prima facie* case... Rebuttal evidence and arguments can be presented in the specification, ... by counsel, ..., or by way of an affidavit or declaration under 37 CFR 1.132...
>
> When considering whether proffered evidence is commensurate in scope with the claimed invention, Office personnel should not require the applicant to show unexpected results over the entire range of properties possessed by a chemical compound or composition.... Evidence that the compound or composition possesses superior and unexpected properties in one of a spectrum of common properties can be sufficient to rebut a *prima facie* case of obviousness.

(Emphasis added).

The Examiner asserted that the 131/132 Declaration is not commensurate in scope with the claims because "the instant claims do not recite any limitations with respect to lipid mixtures or the number of amplicons necessary for the assay" or "there are no limitations requiring specific level of detection" (final Office Action at pages 2-3). However, to the contrary, independent Claim 16 explicitly recites the number of nucleic acid segments (50-1,000). In addition, Claim 37 recites a subattomolar detection level and Claims 44-45 recite phospholipids and a method of encapsulating. Thus, the 131/132 Declaration is commensurate in scope with the pending claims and establishes superior and unexpected detection results of the claimed ILNAA assay.

c. Secondary Considerations Support Patentability

Secondary considerations must also be considered in any obviousness determination. Exhibits 1-6 were attached to the Response filed on January 2, 2007 and are included in the attached Evidence Appendix herein. The Exhibits evidence solution to long-felt but unsolved needs and failure of others. For example:

Science, Vol 309, page 1811:

> Other talks spotlighted new biosensors for detecting chemical and biological agents. Their common goal is to sniff out smaller and smaller doses of toxins in the environment with greater speed and accuracy. For example, a new sensing technique described by Jeffrey Mason, a researcher at the Armed Forces Institute of Pathology in Washington, D.C., can detect as few as 500 molecules of cholera or botulinum in a sample. That's 1000 times more sensitive than existing techniques.

Scientific American.com:

> Biophysicists Jeffrey Mason of the Armed Forces Institute of Pathology, Timothy O'Leary of the Veterans Health Administration and their colleagues paired specific antibodies for botulin and cholera with the crime scene DNA-amplification technique known as polymerase chain reaction, or PCR. By combining the two, the scientists can detect trace amounts of the biological agents in urine samples, water or other mediums. "We can actually detect down to 10 molecules of biotoxin in a sample," Mason notes. "We're always below 500 molecules."
>
> But the test also improves on its predecessors by delivering fewer false positives. Previous attempts to couple antibodies and PCR often led to incorrect results due to DNA contamination from the lab or instruments involved. So the scientists encased the antibody and DNA in a liposome, a manmade fat cell capable of sheltering up to 60 copies of DNA fragments. Since the important DNA is protected, the entire sample can be subjected to rigorous cleaning to remove any DNA contamination, Mason notes.
>
> Previous attempts to couple antibodies and PCR often led to incorrect results due to contamination for the lab or instruments involved . . . The new test is quick and as much as 1,000 more times sensitive in detecting

> cholera or botulin than any other assay, according to the paper presented the research in yesterday's *Nature Biotechnology.*

American Chemical Society (chemistry.org):

> The technique demonstrated exceptional sensitivity, with a detection threshold of 10 toxin molecules in a 150 µL sample.

New York Times, pages 2-3 "Keeping the DNA Clean":

> Jeffrey T. Mason of the Armed Forces Institute of Pathology and colleagues have now devised a PCR test for cholera and botulism that reduces the contamination problem by encapsulating the DNA in liposomes, hollow droplets of fat. Only after the sample has been repeatedly cleaned is the DNA released and amplification takes place. The result is a rapid and sensitive field test that is effective with samples containing as few as 10 toxin molecules.

The Examiner at first merely stated, without any further explanation, that these Exhibits do not overcome the obviousness rejections (final Office Action dated May 6, 2007 at page 8). When Applicants requested clarification, the Examiner then asserted that the exhibits fail to prove evidence that the long-felt need "has been a persistent one." (Office Action dated October 12, 2007 at page 12).

To the contrary, as shown above by a diverse group of publications, the present invention is highlighted as having exceptional results in comparison to "existing techniques", "predecessors [tests]", "previous attempts", and "any other assay". In addition to showing long-felt but unsolved needs, the Exhibits also establish failure of others, most notably that other attempts to combine antibodies and PCR often led to incorrect results due to contamination from the lab or instruments involved. Finally, a nexus between encapsulating or sheltering up to 60 copies of DNA fragments in liposomes and the superior detection results as compared to predecessor attempts and tests is also shown.

Accordingly, Applicants maintain that the Exhibits support the non-obviousness of the present invention and rebut the *prima facie* case of obviousness, along with the incompatibility of Singh et al. for nucleic acid encapsulation and the superior and unexpected detection results demonstrated in the 131/132 Declaration.

Applicants maintain that the Examiner has used the instant patent application as a template for hindsight reconstruction regardless of any factual or scientific evidence presented to the contrary. For all these reasons, it would not have been obvious for one of ordinary skill in the art to practice the claimed method for detecting an analyte with an ILNAA assay, as recited in independent Claim 16.

B. Claims 21-22

The Examiner relies upon the *Conclusions* section of Singh et al. for generally teaching "covalent attachment". In addition, the Examiner states that attachment of proteins to hydrocarbons is "within the ability of a skilled artisan to perform." (final Office Action at page 4).

Singh et al. teaches that liposomes may "offer sites for covalent chemistry" (page 6024, right hand column). However, there is no support for the Examiner's assertion that covalent attachment of proteins (antibodies) to hydrocarbons is well known in the art, much less that it is known to anchor receptors to the surface of the liposomal bilayers through covalent attachment to a long-chain-length hydrocarbon having 12 to 24 carbons, as recited in Claim 21. Thus, the Examiner has failed to provide a rationale identifying a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed invention does.

Moreover, it is axiomatic that a simple statement that modifications of the prior art would have been "within the ordinary skill of the art" because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason

to combine the teachings of the references. Accordingly, in view of the Examiner's unsupported assertions, a *prima facie* case of obviousness has not been demonstrated.

C. Claim 23

Singh et al. explicitly states that because the liposomes "do not contain any protein component, they have vastly superior shelf life than antibody-marker conjugates typically used in immunoassays and biosensors" like Wu et al. (page 6024, right hand column, emphasis added).

Accordingly, Singh et al. *teaches away* from associating receptors to the extravesicular surface of the liposomal bilayers by covalently attaching an antibody (i.e., protein) to glycolipids or phospholipids, as recited in Claim 23.

It is axiomatic that teaching away is a *per se* indication of nonobviousness. One of ordinary skill in the art would have been discouraged from combining Singh et al. and Wu et al. to achieve the claimed invention in view of the express teachings of Singh et al. to avoid antibodies or the antibody-marker conjugates of Wu et al.

D. Claim 38

Neither Singh et al. nor Wu et al. teaches or suggest linking a specific receptor to a liposomal bilayer encapsulating nucleic acid segments having a unique nucleotide length, thereby screening for several target analytes at one time, as recited in Claim 38. In fact, Claim 38 was never discussed by the Examiner in the final Office Action dated June 18, 2008 or either the first or second Advisory Action.

The Examiner has failed to provide a rationale identifying a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed invention does. Accordingly, in view of the Examiner's unsupported assertions, a *prima facie* case of obviousness has not been demonstrated.

E. Claim 43

In the Amendment After Final Rejection dated September 3, 2008, Applicants argued that the Examiner had not cited to any teaching or support to render this method obvious in final Office Action. In the second Advisory Action at page 2, the Examiner states for the first time, without any support, that the spontaneous rupture is an inherent feature of all liposomes when receptors in the bilayer aggregate when exposed to a target analyte.

However, this assertion is contrary to the plain teachings of the cited art. According to Singh et al., in which a signal lipid is incorporated into the bilayer of the liposome, the liposome must be lysed with surfactant Triton X-100 (pages 6022-23). Neither Singh et al. nor Wu et al. teaches or suggests exposing the receptors to a target analyte, causing aggregation of the receptors within the plane of the liposomal bilayers, wherein the aggregation causes the liposomal bilayers to become unstable leading to spontaneous rupture of the liposomal bilayers, as recited in Claim 43. In is axiomatic that inherency cannot be asserted in a vacuum by possibilities and probabilities.

II. The Rejection of Claim 18 over Singh et al. in view of Wu et al. and further in view of Cao et al.

Claim 18 is grouped with Claims 16-17, 21-24, 26-28, 33, 35-38, and 40-41 as argued in section I(A) above and stands or falls with those claims.

III. The Rejection of Claims 20, 42, and 46 over Singh et al. in view of Wu et al. and further in view of Bernstein

A. Claim 20

Claim 20 is grouped with Claims 16-17, 21-24, 26-28, 33, 35-38, and 40-41 as argued in section I(A) above and stands or falls with those claims.

B. Claim 42

Bernstein discloses an assay utilizing a receptor or antibody sensitized liposome particles having ATP encapsulated therein. The assay may detect the presence of analytes such as antigens and DNA probes (Abstract). Bernstein discloses that a DNA probe analyte may be immobilized on a "particle" (col. 5, lines 30-35).

The Examiner does not offer any reason for the combination of Singh et al., Wu et al., and Bernstein other than to state that Bernstein "teaches a particle" (final Office Action dated June 18, 2008 at page 9).

First, the claimed nucleic acid segments are encapsulated. Thus, it is unclear how Bernstein, which requires DNA probes to be immobilized on a particle and then bound to an outside of a liposome, can be combined with either Singh et al. or Wu et al. to achieve the claimed invention. Second, Bernstein does not teach or suggest that an immobilized target analyte is immobilized on magnetic micro-particles or a micro-fabricated device, as recited in Claim 42. The reasoning for the asserted combination of references in such a way as to render the claimed invention obvious has never been clarified by the Examiner.

C. Claim 46

As noted, Singh et al. states that because the liposomes "do not contain any protein component, they have vastly superior shelf life than antibody-marker conjugates typically used in immunoassays and biosensors" like Wu et al. (page 6024, right hand column, emphasis added). Bernstein discloses an assay utilizing an antibody sensitized liposome particles having ATP encapsulated therein (Abstract).

In view of the express teachings of Singh et al. to avoid protein components such as antibodies, one of ordinary skill in the art would not have been motivated to associate monoclonal or polyclonal antibody receptors of Bernstein to the extravesicular surface of the liposomes in Singh et al. Teaching away is a *per se* indication of nonobviousness

and one of ordinary skill in the art would have been led away from the claimed invention in view of the express teachings of Singh et al. to avoid antibodies. Thus, it would not have been obvious for one of ordinary skill in the art to associate monoclonal or polyclonal antibodies to the extravesicular surface of liposomal bilayers, as recited in Claim 46.

IV. The Rejection of Claims 29-30 over Singh et al. in view of Wu et al. and further in view of Boxer et al.

Claims 29-30 are grouped with Claims 16-17, 21-24, 26-28, 33, 35-38, and 40-41 as argued in section I(A) above and stands or falls with those claims.

V. The Rejection of Claim 31 over Singh et al. in view of Wu et al. and further in view of Huang et al.

Huang et al. discloses RNA-PCR studies in which genomic DNA can produce incorrect results because of its potential to act as a secondary competitor for RNA quantitation. Huang et al. discloses using DNAse to remove contaminating DNA from RNA in an RNA-PCR study. There is no physical separation of the DNA and RNA sequences. The entire purpose of Huang et al. is to digest DNA normally present in RNA preparations.

There is no recognition or appreciation in Singh et al., Wu et al., or Huang et al. that liposome-encapsulated nucleic acid segments are protected from enzymatic degradation because a DNAse or RNAse enzyme cannot cross a liposomal bilayer. In fact, the entire purpose for digesting DNA in Huang et al. is not present in the claimed invention because, unlike Huang et al., in the claimed assay the nucleic acid sequences are encapsulated inside closed-shell liposomes and are physically sequestered from the rest of the assay solution comprising background DNA or RNA.

The present invention allows for the addition of DNase or RNase to the assay solution while protecting the nucleic acid sequences in the liposomes that will be

amplified later. Thus, there is no teaching to reduce background DNA or RNA contamination of the assay - the ILNAA assay - by adding DNase or RNase to the assay solution, as recited in Claim 31.

VI. The Rejection of Claims 44-45 over Singh et al. in view of Wu et al. and further in view of Bailey et al.

Claims 44-45 are grouped with Claims 16-17, 21-24, 26-28, 33, 35-38, and 40-41 as argued in section I(A) above and stands or falls with those claims.

VII. CONCLUSION

Applicant respectfully requests the Examiner to reopen prosecution or allow the patent application. Should the Examiner have any questions, comments or suggestions, the Examiner is invited to contact Applicant's representative at the number indicated below.

Respectfully submitted,

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Date: December 3, 2008

CLAIMS APPENDIX

1-15. (Canceled)

16. (Previously Presented) A method for detecting an analyte with an immunoliposome-nucleic acid amplification assay, comprising:

encapsulating 50 to 1,000 identical nucleic acid segments within closed shell liposomal bilayers,

associating receptors to the extravesicular surface of said liposomal bilayers,

exposing the receptors to an immobilized target analyte, which binds to the liposomal bilayer associated receptors;

removing unbound liposomal bilayers;

lysing the bound liposomal bilayers to release the nucleic acid segments;

amplifying the nucleic acid segments released from said liposomal bilayers, and

detecting the amplification products of the nucleic acid segments to quantify the amount of the target analyte.

17. (Previously Presented) The method of claim 16, wherein the target analyte is selected from the group consisting of proteins, nucleic acids, carbohydrates, glycolipids, gangliosides, viruses, bacteria, toxins, chemical warfare agents, explosives, poisons, hormones, cancer-specific soluble biological markers, tumor cell-surface markers, and minor cell components in larger cell populations.

18. (Previously Presented) The method of claim 16, wherein the immunoliposome-nucleic acid amplification assay can be used to spatially localize an analyte within a fresh or fixed tissue section.

19. (Canceled)

20. (Previously Presented) The method of claim 16, wherein the receptors are selected from the group consisting of monoclonal or polyclonal antibodies, antibody Fab' fragments, glycolipids, soluble proteins, dyes, DNA probes, and RNA probes.

21. (Previously Presented) The method of claim 16, comprising anchoring the receptors to the surface of the liposomal bilayers through covalent attachment to a long-chain-length hydrocarbon having 12 to 24 carbons.

22. (Previously Presented) The method of Claim 21, wherein the long-chain-length hydrocarbon comprises carboxylic acids, amines, thiols, alcohols, aldehydes, nitrites, amides, or halides.

23. (Previously Presented) The method of Claim 16, wherein associating receptors to the extravesicular surface of the liposomal bilayers comprises covalently attaching an antibody to glycolipids or phospholipids.

24. (Previously Presented) The method of Claim 16, wherein associating receptors to the extravesicular surface of the liposomal bilayers comprises electrostatically coupling charged receptors to charged lipids in the liposomal bilayers.

25. (Canceled)

26. (Previously Presented) The method of claim 16, comprising anchoring integral membrane protein receptors to the liposomal bilayers by direct incorporation into the liposomal bilayers.

27. (Previously Presented) The method of claim 16, comprising reducing non-specific binding of the liposomal bilayers on an immobilizing substrate by varying the

lipid composition of the liposomal bilayers to alter the size of the liposome, the fluidity of the bilayer, or the polarity and charge of the surface of the liposomal bilayer.

28. (Previously Presented) The method of claim 16, further comprising reducing non-specific binding of the liposomal bilayers on an immobilizing substrate by altering the charge density of the surface of the liposomal bilayer.

29. (Previously Presented) The method of claim 16, further comprising reducing non-specific binding of the liposomal bilayers on an immobilizing substrate by attaching polyethylene glycol to the surface of the liposomal bilayer.

30. (Previously Presented) The method of claim 16, further comprising reducing non-specific binding of the liposomal bilayers on an immobilized substrate by varying the length of a spacer arm used to attach the receptors to the liposomal bilayers.

31. (Previously Presented) The method of claim 16, further comprising reducing background DNA or RNA contamination of the assay by adding DNase or RNase to the assay solution, thereby degrading background DNA or RNA.

32. (Previously Presented) The method of claim 16, wherein the liposomal bilayers are lysed using an alcohol or melittin.

33. (Previously Presented) The method of claim 16, wherein said amplifying comprises polymerase chain reaction, real-time PCR, bDNA or Q-beta replicase methods.

34. (Previously Presented) The method of claim 16, wherein said detecting comprises capillary electrophoresis or spectrophotometric assays using nucleic acid-specific dyes.

35. (Previously Presented) The method of claim 16, wherein said amplifying and detecting are coupled.

36. (Canceled)

37. (Previously Presented) The method of claim 16, wherein the target analyte is detected in subattomolar quantities.

38. (Previously Presented) The method of claim 16, further comprising linking a specific receptor to a liposomal bilayer encapsulating nucleic acid segments having a unique nucleotide length, thereby screening for several target analytes at one time.

39. (Previously Presented) The method of claim 16, comprising detecting toxins in soil, water or air.

40. (Canceled)

41. (Previously Presented) The method of claim 16, comprising detecting target analyte in biological fluids.

42. (Previously Presented) The method of claim 16, wherein the immobilized target analyte is immobilized on magnetic micro-particles or a micro-fabricated device.

43. (Previously Presented) A method for detecting an analyte with an immunoliposome-nucleic acid amplification assay, comprising:

encapsulating a plurality of identical nucleic acid segments within closed shell liposomal bilayers,

incorporating receptors into the outer surface of said liposomal bilayers; and exposing the receptors to a target analyte, causing aggregation of the receptors within the plane of the liposomal bilayers, wherein the aggregation causes the liposomal bilayers to become unstable leading to spontaneous rupture of the liposomal bilayers, and release of the nucleic acid segments.

44. (Previously Presented) A method according to Claim 16, wherein encapsulating the plurality of identical nucleic acid segments within closed shell liposomal bilayers comprises mixing phospholipid single-shell vesicles with ethanol and calcium chloride to form phospholipids-nucleic acid segment complexes and dialyzing said complexes.

45. (Previously Presented) A method according to Claim 43, wherein encapsulating the plurality of identical nucleic acid segments within closed shell liposomal bilayers comprises mixing phospholipid single-shell vesicles with ethanol and calcium chloride to form phospholipids-nucleic acid segment complexes and dialyzing said complexes.

46. (Previously Presented) The method of claim 16, wherein the receptors are monoclonal or polyclonal antibodies.

EVIDENCE APPENDIX

A. Combined Declaration Under 37 C.F.R. 1.131 and 1.132 filed on February 12, 2008 (7 Pages) and considered by Examiner in June 18, 2008 final Office Action. As the 131 section of the Declaration is not relied upon in this Appeal, the exhibits attached to the 131/132 Declaration are not included herewith.

B. Exhibits 1-6 (21 pages) submitted with January 2, 2007 Amendment and considered by Examiner in the March 6, 2007 final Office Action and again in the October 12, 2007 Office Action are included herewith:

1. Science, Vol. 309, 1810-11 (September 16, 2005)
2. Scientific American.com (April 17, 2006)
3. American Chemical Society, chemistry.org (May 1, 2006)
4. New York Times "Observatory" (April 25, 2006)
5. Nature Protocols Vol 1. No. 4. 2003 (2006)
6. Nature Biotechnology, Advance Online Publication (2006) [3]

[3] In a November 19, 2008 telephone conference with Carolyn Johnson, paralegal for the BPAI, she confirmed it was acceptable to include the documents in the Evidence Appendix even though it may result in non-sequential pagination, for example, with the Related Proceedings Appendix.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/759,099	Confirmation No. 4916
Applicant	:	Timothy J. O'Leary et al.	
Filed	:	January 20, 2004	
TC/A.U.	:	1637	
Examiner	:	CALAMITA, H.	
Docket No.	:	AFIP 03-16 01	
Customer No.	:	27370	

For: IMMUNOLIPOSOME-NUCLEIC ACID AMPLIFICATION (ILNAA) ASSAY

COMBINED DECLARATION UNDER 37 C.F.R. 1.131 AND 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Dr. Jeffery Mason and Dr. Timothy O'Leary, hereby declare and state that:

1. We are inventors of the above patent application.

2. We are familiar with the rejection of the pending claims, particularly over Singh et al. (Anal. Chem., November 2000) in view of Wu et al. (Letters in Applied Microbiology, May 2001).

3. These references were discussed in detail at a June 12, 2007 personal Examiner interview attended by us, our representative, Examiner Calamita, and Primary Examiner Strzelecka.

4. The present application is directed to an Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay which includes encapsulating a plurality of identical nucleic acid segments within closed shell liposomal bilayers; associating receptors to the extravesicular surface of said liposomal bilayers; exposing the receptors to an immobilized target analyte, which binds to the liposomal bilayer associated receptors; removing unbound liposomal bilayers; lysing the bound liposomal bilayers to release the

nucleic acid segments; amplifying the nucleic acid segments released from the liposomal bilayers, and detecting the amplification products of the nucleic acid segments to quantify the amount of the target analyte.

I. 1.131 Declaration: Antedating the Singh et al. and Wu et al. References

5. We set forth the following facts to antedate the Singh et al. and Wu et al. references.

6. Attached hereto are 2 redacted letters concerning a grant application for Sensitive Detection of Antigens or Antibodies by Immunoliposome - DNA Amplification Hybrids that was submitted to the US Army Medical Research Institute of Infections Diseases, Fort Detrick, MD. The grant was ultimately not funded. Both letters are dated prior to November 2000. See Exhibits 1-2.

7. A partial redacted copy of the corresponding grant application is also attached. See Exhibit 3.

8. Also attached is a partial copy of the Armed Forces Institute of Pathology internal annual report, which on page 64 under RESEARCH indicates the ongoing "developmental work in immunoliposome-PCR (an ultrasensitive assay system for biological and chemical antigens)". The annual report is dated prior to November 2000. See Exhibit 4.

9. Beginning prior to the November 2000, we and our team diligently worked to reduce the immunoliposome-nucleic acid amplification (ILNAA) assay to practice.

10. As evidenced by the attached Letter of Intent to Mr. Glisson of the U.S. Army Medical Research and Materiel Command dated 20 April 2001, research was ongoing. In the Letter of Intent, Dr. Mason describes a proposal for coupling the selectivity and high binding affinity of antibodies for biological molecules with the near-infinite signal amplification of PCR by encapsulating short DNA sequences (primers) into enclosed

phospholipid membranes (liposomes) and conjugating antibodies specific to biological hazards to the outer liposomal membranes. See Exhibit 5.

11. Research Proposal BAA 99-1 followed the Letter of Intent a month later. See Abstract on page 2 of Exhibit 6.

12. We continued to work on the invention as part of our normal duties for the U.S. Army. At no time did we abandon or stop working on the ILNAA assay other than taking care of other matters that are part of our normal work routines. Our work continued until a working method was completed and reduced to practice in September 2002.

13. After completing a working method, we prepared a Department of the Army Invention Disclosure to advise the U.S. Army of the invention, a partial redacted copy of which is attached. The description of the invention and drawings confirm that we and our team reduced the invention to practice prior to January 10, 2003. See Exhibit 7.

14. The individuals on our team can provide further corroboration that for the period prior to November 2000 and until completion of a working example in September 2002, we were diligent in pursuing reduction to practice of the invention and at no time ceased working on the project or otherwise stopped our efforts during the course of our normal workday.

15. All activities and disclosures described above occurred in the United States of America.

II. 1.132 Declaration

A. The Liposomes of Singh et al. Are Incompatible With DNA Encapsulation

16. Singh et al. uses a lipid mixture (L-α-distearoylphosphatidycholine) and L-α-dimyristoylphosphatidylehtanolamine) that is not compatible with DNA encapsulation.

The extrusion method of Singh et al. for forming liposomes is also not compatible with DNA encapsulation. The lipid mixture and extrusion method only allow for passive encapsulation of DNA segments (amplicons) into the liposomes.

17. There are approximately 220,000 lipid molecules per liposome for the 120 nm-diameter liposomes formed using the membrane extrusion method of Singh et al. Thus, the total lipid concentration yields about 85×10^{12} liposomes in 1 mL of the aqueous buffer. This concentration of liposomes (of bilayer thickness 3.7 nm) yields a total internal volume of about 7.5×10^{-16} mL. The liposomes formed by the method of Singh et al. use the membrane extrusion method, which would passively encapsulate the amount of amplicon present in the solution as the liposomes form. Accordingly, the number of moles of amplicon encapsulated into each liposome would be about 7×10^{-24} moles. This corresponds to only about 4 amplicons per liposome.

18. Four (4) amplicons per liposome is unacceptable for use in the ILNAA assay for the following reasons:

(a) only 6% of the total amplicon in solution will be encapsulated into the liposomes, with 94% being on the outside and lost during the purification of the liposomes; and

(b) 4 amplicons per liposome places the distribution of amplicons in the liposomes into the realm of Poisson statistics. More specifically, for 100 liposomes, one would expect almost 25% of the liposomes to be empty. This is unacceptable for the proper performance of the ILNAA assay. To avoid complications resulting from Poisson statistics, there ideally should be at least 100 amplicons per liposome.

19. In summary, the method of Singh et al. is unsatisfactory for preparing liposomes with encapsulated DNA amplicons for use in the ILNAA assay method. Further, one or ordinary skill in the art would not be able to simply substitute the

rohamine-labeled fluorophores of Singh et al. with the DNA segments of Wu et al. as asserted by the Examiner.

B. Superior Results Obtained With the Claimed ILNAA Assay

20. The present ILNAA application teaches an Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay for achieving a detection limit of 10-1000 molecules of toxin in subattomolar quantites (i.e., below 1×10^{-18} moles). In fact, the immunoassay method of the present invention can achieve a detection limit of 10 molecules of cholera toxin (17×10^{-24} moles or 17 yoctomoles) and 12 molecules of botulinum neurotoxin type A (20 yoctomoles).

21. The method of Singh et al. teaches an assay method with a detection limit of 1×10^{-10} moles or 0.1 nanomoles for botulinum neurotoxin (page 6024).

22. The method of Wu et al. teaches an assay with a detection limit of 33×10^{-18} moles or 18 attomoles for botulinum neurotoxin type A (Abstract).

23. As shown in the Table below, the Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay of the present invention is 5×10^{12} times more sensitive than the method of Singh et al. and 2×10^{6} times more sensitive than the method of Wu et al.

TABLE: COMPARISON OF DETECTION LIMITS

	Detection Limit of Botulinum neurotoxin	Difference In Detection Limit Between ILNAA application and Prior Art
Present Application	20×10^{-24} moles	----
Singh et al.	1×10^{-10} moles	5×10^{12} ILNAA is 5 Trillion (5,000,000,000,000) Times more sensitive
Wu et al.	33×10^{-18} moles	2×10^{6} ILNAA is 2 Million (2,000,000) Times more sensitive

24. The dramatic increase in sensitivity achieved by the method of the present patent application demonstrates the superior properties of the claimed methods over either the fluoroimmunoassay of Singh et al. or the immuno-PCR assay of Wu et al.

25. In addition, the superior sensitivity of the claimed ILNAA cannot be achieved by the collective teachings of Singh et al. and Wu et al. As noted, DNA segments are not compatible with the liposomes of Singh et al. Further, even if these references were properly combinable, which they are not, one of ordinary skill in the art would have at best estimated that any combination would have increased the sensitivity of the Singh et al. assay to be close to the attomolar level (10^{-18}) of Wu et al., nowhere close to the yoctomolar (10^{-24}) level achieved by the present invention.

IV. VERIFICATION

26. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jeffrey G Mason

Dr. Jeffrey Mason — 11 February 2008 Date

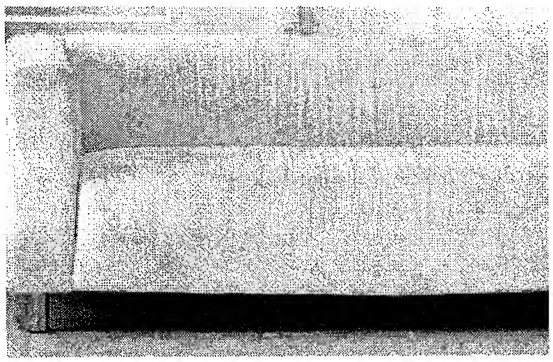
Timothy J. O'Leary

Dr. Timothy J. O'Leary — 11 February 2008 Date

WASHINGTON, D.C.—About 13,000 chemists, physicists, and engineers gathered here from 28 August to 1 September to discuss research with applications including environmental protection, national security, and future energy sources.

Safer Alternative Could Replace Widespread Contaminant

Stain-resistant carpets, upholstery, and fabrics have a dark underside. A common coating that keeps them pristine has recently been found to break down into perfluorooctanoic acid, also known as PFOA or C8, a persistent compound that accumulates inside the body and has been fingered as a possible carcinogen. Manufacturers have been scrambling to come up with alternatives, but none could rival C8-producing stain fighters. At the American Chemical Society (ACS) meeting, however, chemists from the University of North Carolina, Chapel Hill (UNC-CH), unveiled an alternative that repels stains with the best of them but that breaks down into compounds that don't accumulate in the body.



Green clean. New polymers resist stains without breaking down into persistent compounds.

"It's a great step forward," says Tim Kropp, a toxicologist with the Environmental Working Group in Washington, D.C., who has closely followed C8 health concerns. Kropp notes that C8 is found in the blood of 96% of Americans and has been detected everywhere from the middle of the Pacific and Atlantic oceans to embedded in Arctic ice. Animal tests have suggested that the compound is a potential carcinogen, although that has yet to be confirmed in people. Still, the persistence of C8 has persuaded Canada to ban some of the compounds that break down to form C8 in the environment. C8 is also an industrial solvent in its own right, and manufacturers have begun to switch to other solvents and phase out its use. But many researchers suspect that textile and paper coatings, which are ubiquitous, are the largest environmental source of the chemical.

Current polymer fabric coatings owe their popularity to fluorine, an element that when added to polymers makes them strongly repel both water and oil. The polymers consist of a long hydrocarbon backbone bristling with innumerable fluorine-containing arms, each containing eight carbons. Over time, the arms can break off and react with oxygen to form C8. That compound has a combination of size and chemical behavior that makes it readily taken up in the body but difficult for the body to break down and eliminate, says Joseph DeSimone, a UNC-CH chemist who led the effort to develop the new alternative.

DeSimone says that about 2 years ago, he and Paul Resnick, a polymer chemist formerly with DuPont and now at UNC-CH, noticed animal studies that suggested that fluorinated hydrocarbons with four instead of eight carbon atoms in the chains don't persist in the body. So they set out to make one with good stain-resistant qualities. Researchers at 3M had commercialized fluoropolymers with four carbons in the side chains for use as manufacturing solvents. But those compounds, the UNC-CH researchers found, did not repel water and oil as well as the longer chain compounds did. Part of the problem, DeSimone notes, is that the shorter side chains don't pack tightly around the hydrocarbon backbone. As a result, the backbone can more easily interact with oil and water, thereby making the chemicals less repellent.

To get around this problem, Ji Guo, a Ph.D. student in DeSimone's lab, doctored the C4 side chains, outfitting each with an extra pair of hydrocarbon groups called methylenes. The methylenes, DeSimone says, encouraged the side chains to pack tightly together, making a more formidable barrier around the hydrocarbon backbone. Tests of the new materials showed that they repel oil and water almost identically to the longer-side-chained polymers, Guo says. But because the new coatings are made from polymers with shorter side chains, even if they break down over time, there is no way that they can generate C8. DeSimone says he and his colleagues have applied for patents on the new materials and have already had several discussions with textile manufacturers interested in the technology. Kropp says the new compounds must be tested to make sure there are no unforeseen problems. However, he adds, "it's always great to see scientists come up with an alternative to a problematic compound."

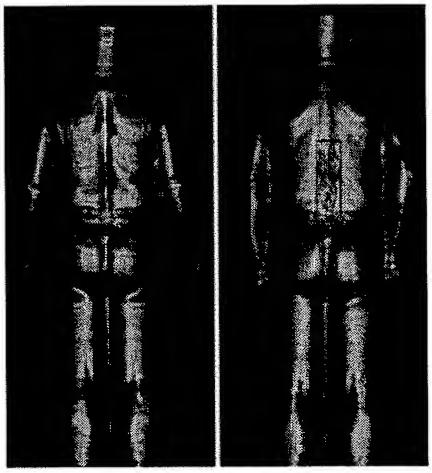
–ROBERT F. SERVICE

New Techniques Aim To Thwart Terrorists

In more than a dozen sessions at the ACS meeting dedicated to defense and homeland security, researchers presented technologies aimed at countering every imaginable terrorist threat—from devices for sensing explosives strapped onto the body of a suicide bomber to sensors capable of detecting microscopic quantities of biotoxins injected into a city's water supply.

Not surprisingly, many talks focused on transportation security. The tools currently available to screeners at airports and subway stations—metal detectors, x-ray scanners, sniffer dogs, and manual pat-downs—can't detect explosives or nonmetallic weapons concealed inside luggage or on the body of a passenger. Two technologies presented at the meeting offer a solution to those problems, although they both have a way to go before they can be deployed.

One, developed by David Sheen and his colleagues at Pacific Northwest National Laboratory in Seattle, Washington, uses electromagnetic radiation of millimeter wavelength to see through clothing and other barriers. Ranging between 30 and 300 gigahertz in fre-



Nailed. Millimeter waves spot plastic explosive strapped to a tester's spine (*right*).

CREDITS (TOP TO BOTTOM): JUPITER IMAGES; JIM ZIV

Published by AAAS

quency, these are the same microwaves used for applications such as wireless access to the Internet. Different materials on a person's body reflect them to varying degrees, enabling a computer to generate a three-dimensional image showing the outlines of concealed objects. Because the waves are nonionizing, "they do not pose any health risks," Sheen says. The scan currently takes up to 10 seconds, during which the person must stand relatively still. Generating the image takes up to another 30 seconds. Sheen says his group is working to speed up the system.

A similar technology described by Robert Barat, a chemical engineer at the New Jersey Institute of Technology in Newark, uses waves of a shorter wavelength. Submillimeter (or terahertz) waves, familiar to radio astronomers, generate a spectrum when they interact with a material. They can also be transmitted farther than millimeter waves can. By harnessing those properties, Barat's group hopes to design scanners that would be capable of detecting weapons and bombs carried by a terrorist more than 5 meters away. The method "has the potential of not only showing the presence of a hidden substance but also of identifying the substance based on a transmission or reflection spectrum," says Barat, who has yet to build a prototype. Jehuda Yinon, an expert on explosives detection at the Weizmann Institute of Science in Rehovot, Israel, says the technology could be an invaluable tool for identifying suicide bombers in public places.

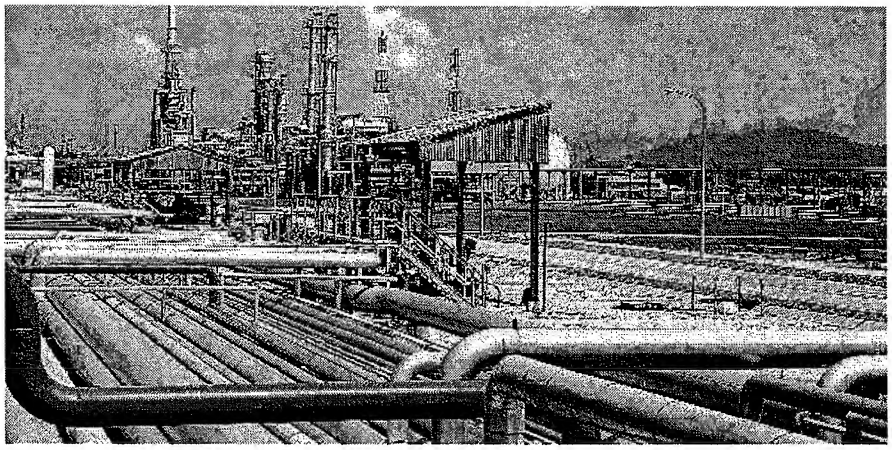
Other talks spotlighted new biosensors for detecting chemical and biological agents. Their common goal is to sniff out smaller and smaller doses of toxins in the environment with greater speed and accuracy. For example, a new sensing technique described by Jeffrey Mason, a researcher at the Armed Forces Institute of Pathology in Washington, D.C., can detect as few as 500 molecules of cholera or botulinum in a sample. That's 1000 times more sensitive than existing techniques.

The heart of the sensing device is a liposome—a molecular cylinder made up of lipids—with a DNA molecule encapsulated inside and a receptor molecule on the outside that attaches specifically to the toxin. The toxin molecules are first captured on a plate using antibodies that bind to the toxin. When the liposomes are added to this mix, the receptor molecules linked to them attach to the toxin as well. At the end of the assay, everything else is washed away, leaving only the liposomes that have been chained to the toxin molecules.

The researchers then split the liposomes open with an enzyme to release the DNA molecules and tally them with a standard polymerase chain reaction (PCR)—in effect, using the DNA molecules as a proxy for the toxin. And because PCR can detect tiny amounts of DNA (by making many copies of DNA molecules present in a sample), the technique can sense extremely low concentrations of toxin. "What they've done is amplified the signal. It's really very clever," says James Robertson, a research biologist at the Federal Bureau of Investigation Laboratory in Quantico, Virginia.

–YUDHIJIT BHATTACHARJEE

CREDIT: JUPITER IMAGES



Cheaper gas? Converting waste into H_2 could lower refining costs and spur a hydrogen economy.

New Routes Toward Practical Hydrogen?

Hydrogen makes a tantalizing fuel. Water is its only byproduct when burned or run through a fuel cell to make electricity. It's also the most abundant element in the universe. But the downside is that earthly hydrogen is almost always bound to other elements, and liberating it requires much more energy than it releases as a fuel. At the meeting, two separate teams reported novel approaches to extracting hydrogen from waste products that could bring a sustainable hydrogen economy a step closer.

In the first, researchers from Pennsylvania and Georgia reported on a new catalyst that converts hydrogen sulfide (H_2S)—an abundant contaminant in natural gas wells—to hydrogen gas (H_2). In the other, researchers from Indiana revealed a new process for recovering H_2 from silicon-based compounds, which could open the door to new ways of generating and storing hydrogen.

Outsiders say it's too early to tell whether these approaches make economic sense. But they are "promising avenues," says Joseph Sadighi, a catalyst expert at the Massachusetts Institute of Technology in Cambridge.

Raiding industrial waste for useful chemicals is nothing new. H_2S is routinely converted to sulfur dioxide (SO_2) as part of a process to generate sulfuric acid, a widely used compound in the chemical industry. But although that reaction turns the sulfur in H_2S into a valuable commodity, it misses an opportunity to do the same for hydrogen by instead converting it to water.

Using vanadium-based catalysts to convert H_2S into SO_2 can generate H_2 instead of water, report Israel Wachs of Lehigh University in Bethlehem, Pennsylvania, and Andrew Gibson, who heads Gibson Technologies in Atlanta, Georgia. The conversion, Gibson explained, takes place in two steps. First, carbon monoxide (CO) reacts with H_2S using a long-known reaction to generate H_2 and another compound called carbonyl sulfide (COS), a toxic byproduct. The COS is then fed to another chamber, where it reacts with oxygen over a vanadium oxide catalyst to form SO_2 and CO. The CO is then fed back into the first reaction to generate more H_2.

Unlike the current technology used to convert H_2S to H_2, which extracts the CO needed for the hydrogen-generating reaction from expensive natural gas, the new approach continually generates CO by breaking down the toxic COS. Gibson notes that the process not only might fuel a future hydrogen economy but also could reduce the cost of refining gasoline by supplying H_2 needed to strip crude oil of sulfur.

Purdue University chemist Mahdi Abu-Omar and colleagues offered a very different scheme for generating hydrogen. They discovered it while looking for novel catalysts to convert organic silicon-based liquids called organosilanes into silanols, a more valuable class of compounds used in the chemical industry. The researchers were working with rhenium-based catalysts, which they added to organosilanes and water. They found that the rhenium catalysts not only readily converted their organosilanes into silanols but also generated large amounts of H_2. Organosilanes may make an attractive way to store hydrogen for later use in fuel cells, Omar notes, because both they and the silanol "wastes" are liquids and easy to transport.

Abu-Omar acknowledges that the compounds are somewhat costly to produce and are generated industrially in only small quantities. At the meeting, Sadighi noted that related catalysts might also react with another silicon-based liquid, called PMHS, which is produced in large quantities as a byproduct of the silicone business. Turning this or other more abundant organic compounds into hydrogen could make hydrogen an even more tantalizing fuel.

–ROBERT F. SERVICE

Published by AAAS

SCIENTIFIC AMERICAN.COM

April 17, 2006

More Sensitive, Quicker Test Developed for Cholera and Botulism

The current test for botulin--the potent neurotoxin responsible for paralyzing botulism--involves injecting a mouse with a suspicious sample and waiting to see if it dies. The test is crude, imprecise and can take up to three days to deliver results. But after 72 hours death may have already come for victims of the deadly toxin. Now researchers have discovered an exquisitely sensitive test that kills no animals and takes just three hours to complete.

Biophysicists Jeffrey Mason of the Armed Forces Institute of Pathology, Timothy O'Leary of the Veterans Health Administration and their colleagues paired specific antibodies for botulin and cholera with the crime scene DNA-amplification technique known as polymerase chain reaction, or PCR. By combining the two, the scientists can detect trace amounts of the biological agents in urine samples, water or other mediums. "We can actually detect down to 10 molecules of biotoxin in a sample," Mason notes. "We're always below 500 molecules."

But the test also improves on its predecessors by delivering fewer false positives. Previous attempts to couple antibodies and PCR often led to incorrect results due to DNA contamination from the lab or instruments involved. So the scientists encased the antibody and DNA in a liposome, a man-made fat cell capable of sheltering up to 60 copies of DNA fragments. Since the important DNA is protected, the entire sample can be subjected to rigorous cleaning to remove any DNA contamination, Mason notes.

The new test is quick and as much as 1,000 times more sensitive in detecting cholera or botulin than any other assay, according to the paper presenting the research in yesterday's *Nature Biotechnology.* "The goal is to develop something small

and portable--a lab on a chip version," Mason adds.

It also holds wider promise: if antibodies can be found for other biological agents, like the poison ricin, the same technique could be employed. And, by modifying the liposome, it could even potentially be useful in early detection of disease. "The goal is to use this to detect disease biomarkers in [the bloodstream], particularly cancer," Mason says. "We're able to check such low levels that this would be a very powerful technique, because most cancer markers are only present in very low concentrations."

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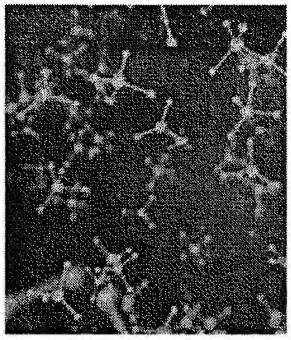
Detection of biological toxins is an extremely challenging technical problem of great societal importance. A very small concentration of a botulin toxin introduced into a city's water supply could be a serious health threat because of the toxin's potency.



PhotoDisc

Such difficult analytical problems can be solved by amplifying the signal, from a blip easily drowned out by all of the other constituents in a sample, to a strong peak that stands out clearly. Fourier transform instruments do this by acquiring signals repeatedly and adding them together. Geneticists use a tactic to amplify a gene fragment in preparation for sequencing: The polymerase chain reaction (PCR) exploits a natural enzyme, polymerase, that can recognize a specific fragment of DNA and then recruit cellular machinery to synthesize multiple copies of it.

In an April 16 advanced online publication to *Nature Biotechnology*, researchers at the Armed Forces Institute of Pathology, the Veterans Health Administration, and the US Food and Drug Administration describe a technique that uses a modified PCR—liposome PCR—to produce an ultrasensitive bioassay capable of detecting biotoxins at just a few molecules per sample.

The work uses PCR to amplify signals, with double-stranded DNA molecules locked within cell-like spheres called liposomes. Each liposome is formed from a bilayer of lipids that line up head to head to produce a thin shell with the hydrophilic (water-soluble) heads sandwiched between the hydrophobic (greasy, water insoluble) tails that line the exterior and interior surfaces of the bilayer. The researchers incorporated about 2,500 molecules of monosialoganglioside GM1into the bilayer. These molecules bind cholera toxin beta subunit (CTBS).

To analyze for CTBS, the researchers first prepared a microtiter plate by attaching CTBS-specific antibodies to the surface of the wells to capture any toxin in a test solution. Next, they added the liposome reagent and incubated for 1 hour to allow the monosialoganglioside GM1 on the liposome surface to bind to any cholera toxin held fast by the antibodies on the well surface. Finally, they added a reagent to rupture the bound liposomes and release the double-stranded DNA. They took an aliquot from each well and performed PCR to confirm the presence or absence of DNA. If a cholera toxin was captured by an antibody lining the wall of a well and then targeted by a liposome, then the liposome's DNA—amplified by PCR—would be a clear indicator of the presence of the toxin.

The technique demonstrated exceptional sensitivity, with a detection threshold of 10 toxin molecules in a 150-µL sample. In a more realistic test, the researchers spiked human urine and farm run-off water with CTBS and noted a threshold of about 400 molecules of CTBS for water and about 40 molecules for urine. The results are 2–3 orders of magnitude more sensitive than the most sensitive assays currently used. Another assay for botulism toxin yielded similar results.

The technique could be improved by incorporating antibodies into the lipid bilayer and then coding the DNA sequence reporter to match the antibody on the liposome's surface; this would allow simultaneous analysis of multiple antigens. The researchers are currently working on such an approach to detect additional chemical and biological warfare agents, as well as to discover biomarkers for cancer and other diseases.

This article first appeared on May 1, 2006.

The New York Times
nytimes.com

April 25, 2006
OBSERVATORY

Pumping Up, the Land Crab Way

By HENRY FOUNTAIN

To shed its shell for a new one, a crab needs a swelled head. And a swelled torso, legs and claws, for that matter. Expanding the body makes the old exoskeleton crack, so the crab can worm its way out of it. But then the crab needs to keep expanding so the new soft shell it secretes is bigger than the old one. Molting is all about growth, after all.

Aquatic crabs expand by taking in more water, creating a temporary hydrostatic "skeleton" of pressurized water that supports the body while the new shell hardens. But what about crabs that spend their time on land?

Jennifer R. A. Taylor, a doctoral student at the University of North Carolina, and her adviser, William M. Kier, have discovered that one land crab, at least, uses air. But it doesn't just pump itself up like a ball; it combines air pressure with water pressure.

"It's kind of like blowing up a balloon inside the body," Ms. Taylor said.

She and Dr. Kier studied red land crabs, Gecarcinus lateralis, which are found in the Caribbean and other tropical regions. They measured the pressure within the crab at various points in the molting process. Their findings were published in Nature.

These crabs, whose bodies are about three inches wide, can take up only small amounts of water when they are on moist sediments. So to molt, the crab takes in air, trapping it in a cavity right behind the head. This

inflated gut then puts pressure on the hemolymph, the bloodlike fluid within the crab.

Because crabs have an open circulatory system, pressurizing the hemolymph causes expansion throughout the body and provides the stiffness and support the crab needs while the shell hardens.

How do the crabs take in the air? The researchers suspect they swallow it, which is what many molting insects do.

While this pneumo-hydrostatic skeleton (as the researchers call it) provides support, it makes the crab something less than a spring chicken, lacking its normal agility. "The crab's body is designed to work as a rigid system," Ms. Taylor said. During molting, she added, "it is less efficient because that same design is being used as a hydrostatic system."

The lack of agility may also be a behavioral response, she said. The more the crab moves, the greater the risk that some deformation will become permanent as the shell hardens.

"Lots of crabs live on land to some extent," Ms. Taylor said, adding that as these crustaceans became more terrestrial, "using air rather than water would be more important."

Keeping the DNA Clean

With potential bioterrorism agents like the bacterial toxins that cause cholera and botulism, a little can go a long way. That raises problems for biologists devising ways to detect them. How do you find tiny amounts of toxin in food, say, or water?

One approach is to link the toxin to fragments of DNA and then, using the lab technique called PCR, or polymerase chain reaction, amplify the fragments until there are enough to easily detect. But PCR requires that samples be kept extremely clean; contamination by other DNA makes the test less sensitive.

Jeffrey T. Mason of the Armed Forces Institute of Pathology and colleagues have now devised a PCR test for cholera and botulism that reduces the contamination problem by encapsulating the DNA in liposomes, hollow droplets of fat. Only after the sample has been repeatedly cleaned is the DNA released and amplification takes place. The result is a rapid and sensitive field test that is effective with samples containing as few as 10 toxin molecules.

Here's how the process works, as described in a paper in Nature Biotechnology. Monoclonal antibodies tailored for a particular toxin are put in the well of a testing plate, where they accumulate on the surfaces. A solution containing the toxin is added, and the toxin molecules bind with the antibodies.

Then the liposomes are added. Each liposome's hollow center contains about 60 copies of a DNA fragment, while the exterior is coated in another toxin-linking molecule. This binds the liposome to the toxin, and the liposome-toxin-antibody combinations stay put while everything else is washed away.

Adding a simple detergent breaks the liposomes open, releasing the DNA, which is then replicated in a process called real-time PCR, producing a fluorescent signal that can be detected. The whole process takes about three hours.

The Right Mix for Cells

Culturing cells in the lab — tumor cells, perhaps, or stem cells — is normally done on a flat surface like glass. But the real world exists in three dimensions, not two. Because cells respond to cues from surrounding cells, it makes sense that the response may be more natural, and the resulting tissue may better mimic the real world, if cells are surrounded in three dimensions rather than two.

But building a three-dimensional matrix for culturing tissue is not easy. A new technique has been developed by Sageeta N. Bhatia and Dirk R. Albrecht of the Massachusetts Institute of Technology and colleagues.

As described in the journal Nature Methods, the technique uses a gel that sets by ultraviolet light.

If you are making a chocolate chip cake, the chips will all settle to the bottom unless you distribute them by stirring so they can be locked in place when the cake bakes. Similarly, in their 3-D culturing technique, the researchers had to come up with a way to distribute the cells — cartilage cells from cows — in the liquid gel before it sets.

The method they came up with was to put the cells in a layer of the gel solution sandwiched between two electrodes. The electrodes create nonuniform electric fields in various patterns that can propel the cells sideways and upward, even though they are not electrically charged.

Once the cells are precisely distributed, they are locked in place by formation of the gel. The gel allows nutrient transport, so the technique may prove to be a good way to study the growth of certain tissues under more lifelike conditions.

An Encouraging Sight

A group of North Pacific right whales has been spotted in the Bering Sea. That's good news for those concerned about this species, perhaps the most endangered whale species in the world.

The whales were nearly hunted to extinction in the 1800's and again in the 1960's. Since then, at most six had been seen in any one year.

But in August and September 2004 sonobuoys picked up calls from whales, which were photographed and in one case, tagged with a radio device. In all 17 whales were spotted, including 7 females and 2 calves. The sightings offer hope that the species may be able to recover.

Liposome polymerase chain reaction assay for the sub-attomolar detection of cholera toxin and botulinum neurotoxin type A

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We describe an ultrasensitive immunoassay for detecting biotoxins that uses a liposome with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as the detection reagent. After immobilization of the target biotoxin by a capture antibody and co-binding of the detection reagent, the liposomes are ruptured to release the reporters, which are quantified by real-time polymerase chain reaction. The new assays for cholera and botulinum toxins are several orders of magnitude more sensitive than current detection methods. A single 96-well microtiter plate can analyze ~20 specimens, including calibration standards and controls, with all measurements conducted in triplicate. Using pre-coated and blocked microtiter plates, and pre-prepared liposome reagents, a liposome polymerase chain reaction assay can be carried out in about 6 h.

INTRODUCTION

The potential use of biological toxins as weapons of mass destruction has created an imperative to develop rapid, field-deployable and highly sensitive assays for the detection of these agents. In addition, assays for biological toxins have applications in such diverse fields as microbiology, clinical diagnostics, the evaluation of therapeutic agents, agriculture and environmental and food testing. Biological toxins typically exhibit extreme potency. For example, botulinum neurotoxin type A (BoNT/A), which is produced by the anaerobic bacterium *Clostridium botulinum*, is about 100 billion times more toxic than cyanide; it is the most lethal human toxin known, with an LD_{50} of approximately ng per kg[1] (ref. 1). Thus, assays for biological toxins must be not only highly specific, but also highly sensitive with the ability, in some applications, to detect toxins down to the level of a few hundred molecules. The only current assay technology capable of this level of sensitivity couples the protein detection specificity of antibody–protein binding with the powerful amplification capability of the polymerase chain reaction (PCR). Immuno-PCR, first introduced by Sano *et al.*[2], uses a reporter oligonucleotide that is either covalently[2] or non-covalently[3] attached to an antibody specific for the protein of interest. Although these methods allow for the highly specific and sensitive detection of protein targets, they have limitations that have prevented their widespread use. These shortcomings include poor sensitivity and reproducibility with complex environmental or biological specimens, the expense and short shelf life of the reagents, the complex synthesis necessary to fabricate the detection reagents and the high susceptibility of the assay to contamination with reporter oligonucleotide. Here we describe an ultrasensitive assay for the detection of biotoxins, which we call liposome polymerase chain reaction (LPCR)[4], that overcomes many of the limitations of conventional immuno-PCR. This assay uses a liposome with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as a detection reagent (**Fig. 1**). After immobilization of the target biotoxin in a microtiter plate well by a capture antibody and co-binding of the DNA-liposome detection reagent, the vesicles are ruptured to release the DNA reporters,

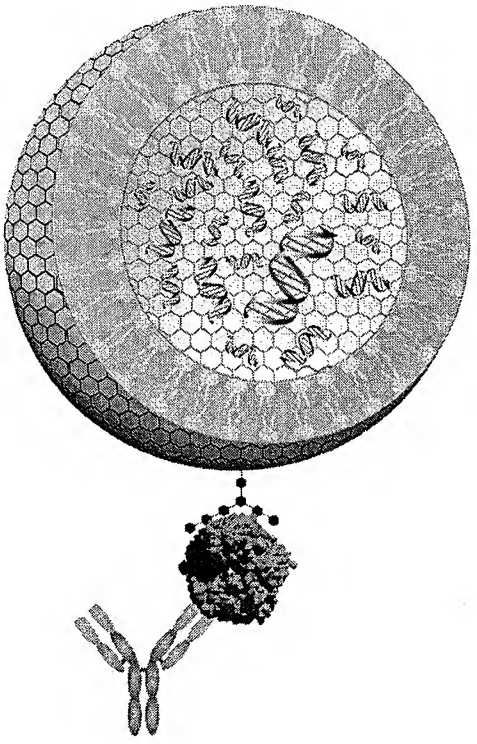

Figure 1 | Representation of a liposome detection reagent shown in cross section. The dsDNA reporters (green with red bars) are encapsulated inside the lipid bilayer of the liposome (yellow) into which monosialoganglioside G_{M1} receptors (blue) have been incorporated. The liposome is shown bound to a cholera toxin beta subunit (CTBS) pentamer, which is co-bound to a capture antibody.

which are quantified by real-time PCR. Encapsulation of reporters inside liposomes offers two advantages over current immuno-PCR methods. First, ~60 reporters can be encapsulated inside each liposome increasing the sensitivity of the assay. Second, any contaminating reporter DNA in the plate wells can be degraded by DNase digestion immediately prior to lysis of the liposomes, as the enzyme cannot pass through the liposomal bilayer. Limitations of LPCR relative to the simpler, but less sensitive, enzyme-linked immunosorbent assay (ELISA) include the need to prepare the DNA-liposome detection reagent and the requirement of performing real-time PCR. LPCR assays for cholera toxin beta subunit (CTBS) and BoNT/A yield detection thresholds below 1 fg ml^{-1}, which is 2–3 orders of magnitude more sensitive than current detection methods[5–7]. Other biotoxins that could potentially be detected using ganglioside receptors include tetanus, pertussis, shiga, ricin and heat-labile enterotoxin[8,9]. We are currently developing LPCR assays that employ antibodies as antigen receptors in place of gangliosides. These assays are being used to detect additional biotoxins as well as biomarkers for cancer, prion disease, dengue virus and human immunodeficiency virus.

MATERIALS

REAGENTS

- Monosialoganglioside G_{M1} from bovine brain (Sigma)
- Cholera toxin beta subunit (non-toxic subunit) from *Vibro cholerae* (CTBS; Sigma)
- Ficoll, 70 kDa (Sigma)
- DNase I from bovine pancreas (Type IV; Sigma)
- Exonuclease III from *Escherichia coli* (Sigma)
- Triton X-100, ultra grade (Sigma)
- Bovine serum albumin, RIA grade (BSA; Sigma)
- Sepharose CL-4B (Sigma)
- Octyl-β-D-glucopyranoside (Sigma)
- Trisialoganglioside $G_{T_{1b}}$ from bovine brain (Calbiochem)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine in chloroform (DOPC; Avanti Polar Lipids) **! CAUTION** Chloroform is a carcinogen and can cause liver and kidney damage. It should be handled and disposed of appropriately (see www.osha.gov for further information).
- Lissamine rhodamine B 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE; Invitrogen/Molecular Probes)
- Anti-cholera toxin beta subunit mouse monoclonal antibody (anti-CTBS; Biodesign Laboratories)
- Botulinum neurotoxin type A from *Clostridium botulinum* (BoNT/A; Metabiologics) **! CAUTION** BoNT/A is extremely toxic with a human LD_{50} of ~1 ng/kg. Only trained personnel should work with this toxin, and registration with the Center for Disease Control and Prevention (CDC) may be required (http://www.cdc.gov). Appropriate laboratory safety procedures should be employed. See the following website and references contained therein for additional details (http://pathema.tigr.org/pathema/BoNT_protocols.shtml).
- Affinity-purified polyclonal rabbit IgG antibody against BoNT/A (anti-BoNT/A; Metabiologics)
- Costar flat-bottom EIA and Easy Wash high-binding 96-well microtiter plates (Fisher Scientific)
- PCR primers (DNA Technologies)
- AmpliTaq Gold DNA polymerase (Applied Biosystems)
- Taqman universal PCR mastermix (Applied Biosystems)
- PCR Taqman probe (Applied Biosystems)
- 10× PCR buffer (Invitrogen)
- TOPO TA cloning kit with pCR2.1-TOPO T/A plasmid vector and One-Shot *E. coli* (Invitrogen)
- TRizol Plus RNA purification kit (Invitrogen)
- SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen)
- Hela cells (ATCC)
- Plasmid DNA Mini-Prep kit (QIAGEN)
- TO-PRO-1 DNA intercalating fluorescent dye (Invitrogen/Molecular Probes)
- QIAquick PCR purification kit (QIAGEN)
- 3M sodium acetate (Sigma)
- Glycogen, ultrapure (Sigma)
- Ethanol, absolute (Aldrich)

REAGENTS SETUP

Coating buffer 50 mM carbonate/bicarbonate buffer (Kirkegaard & Perry), pH 9.6

Blocking/dilution buffer 1% (w/v) BSA in PBS, pH 7.8

Wash buffer A 2 mM imidazol/0.02% (w/v) Tween-20 in PBS (Kirkegaard & Perry), pH 7.4

Wash buffer B PBS, pH 7.4

Digestion buffer 10 mM $CaCl_2$/10 mM $MgCl_2$/20 mM HEPES (Sigma), pH 7.8

Lysis buffer 10 mM Triton X-100 in 10 mM borate (Sigma), pH 9.0

EQUIPMENT

- Probe-tip sonicator, Sonic Dismembrator/model 500, with 1/8" probe (Fisher Scientific)
- Dynamic light-scattering spectrometer, Nicomp/model 370 (Particle Sizing Systems)
- Microtiter plate washer, model ELx405 (Bio-Tek)
- ABI PRISM genetic sequencer, model 7700 (Applied Biosystems)
- GeneAmp 9600 for reporter amplification (Perkin-Elmer Corporation)
- Eppendorf model 5417R bench-top centrifuge with model FA 45-24-11 rotor.
- Optima TLX ultracentrifuge and model TLS 55 swinging bucket rotor (Beckman Instruments)

PROCEDURE

Preparation of reporter

1| The double-stranded DNA (reporter) that is encapsulated inside the liposomes serves only as a PCR amplification surrogate for detection and quantification of the corresponding biotoxin target of the assay. Thus, any convenient sequence can be used; however, the sequence should be <100 bp in length to ensure the best amplification efficiency, and to maximize the number of reporters encapsulated into the liposomes. The reporter should also be a sequence not likely to be found in the specimens to be analyzed.

2| We use an 84-base segment derived from the human β_2-microglobin transcript. Since the final assay does not involve a reverse-transcriptase step, this sequence, which spans an intron, will not be found in contaminating human DNA. The following is the complete sequence of the β_2-microglobin reporter used in this assay[10]: 5′–TGA CTT TGT CAC AGC CCA AGA TAG TAA GTG GGA TCG AGA CAT GTA AGC AGC ATC ATG GAG GTT TGA AGA TGC CGC ATT TGG ATT–3′

3| Pellet HeLa cells suspended in PBS buffer, pH 7.4, by centrifugation at 300*g* for 10 min to produce a pellet of ~1×10^7 cells. Extract the total RNA from the cell pellet using the TRizol Plus RNA Purification kit by following the manufacturer's instructions.

4| Convert the RNA isolated from the cell pellet to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (with random primers) by following the manufacturer's instructions.

5| Prepare reporter DNA by first amplifying cDNA from β_2-microglobin transcripts derived from the HeLa cells using β_2M-246F and β_2M-330R primers (*vide infra*). All primer and probe designs were performed using Taqman Probe & Primer Design software (ABI). The primer sequences are:

β_2M−246F(forward) : 5′-TGA CTT TGT CAC AGC CCA AGA TA-3′

β_2M−330R(reverse) : 5′-AAT CCA AAT GCG GCA TCT TC-3′

Check for the presence of reporter DNA by agarose gel electrophoresis.

6| Clone the amplified reporter into a pCR2.1-TOPO T/A plasmid vector and use this vector to transform One-Shot *E. coli* using the TOPO TA cloning kit by following the manufacturer's instructions.

7| Extract the plasmid DNA using the Plasmid DNA Mini-Prep kit. Amplify a 328 bp DNA fragment from the above recombinant plasmid using M13 forward and reverse primers. This is done to ensure that only the β_2-microglobin reporter is amplified in the final PCR step (*vide infra*). The M13 primer sequences are:

Forward : 5′-GTA AAA CGA CGG CCA G-3′

Reverse : 5′-CAG GAA ACA GCT ATG AC-3′

8| Amplify the DNA fragment using a protocol of 29 cycles as follows:

Cycle number	Denature	Anneal	Extend
1–25	95 °C for 60 s	55 °C for 1 min	72 °C for 3 min
26			72 °C for 10 min

Use a PCR reaction mixture consisting of

Plasmid DNA	10 ng
10× PCR Buffer	5 μl
10 mM dNTP mix	1 μl
25 mM $MgCl_2$	5 μl
M13 forward primer (0.1 μg ml^{-1})	1 μl
M13 reverse primer (0.1 μg ml^{-1})	1 μl
Nuclease-free water	35.6 μl
Taq polymerase (1 unit μl^{-1})	0.4 μl

Following PCR, confirm the presence of the 328 bp DNA fragment by agarose gel electrophoresis.

9| Generate the 85-bp reporter by amplifying the 328-bp fragment using the β_2M-246F and β_2M-330R primer set (15 μM each) using the same PCR conditions as above. Following PCR, confirm the presence of the 85-bp DNA fragment by agarose gel electrophoresis.

10| Purify the 85-bp reporter using a QIAquick PCR purification kit and then precipitate the reporter at −20 °C overnight by adding 1/10 (v/v) of 3M sodium acetate, pH 5.2, and three volumes of absolute ethanol containing glycogen (1 ng ml^{-1}) as a carrier.

11| Centrifuge the DNA solution at 16,000*g* for 25 min at 23 °C. Wash the DNA pellet with 70% ethanol and dry it under a stream of nitrogen. Confirm the purity of the reporter by agarose gel electrophoresis.

12| Dissolve the dry reporter in 500 μl of 10 mM Tris, pH 7.4. Determine the reporter concentration by measuring the DNA solution absorbance at a wavelength of 260 nm. An absorbance of 1.0 at 260 nm corresponds to a reporter concentration of 50 μg ml^{-1}. The weight concentration of the reporter can be converted into molar concentration using the reporter molecular weight, which is 54.9 kDa.

13| Finally, dilute the reporter to a concentration of 667 μg ml^{-1} and store at −80 °C.

■ **PAUSE POINT** The reporter is stable for at least 2 years at this temperature.

An alternative to preparing your own reporter is to purchase it commercially. There are numerous vendors (e.g., Integrated DNA Technologies) that offer synthetic PAGE-purified oligonucleotides of any desired sequence and length. A second option is to purchase an optimized set consisting of a reporter, forward and reverse primers and Taqman probe. Applied Biosystems is one commercial source of such optimized reagents. This approach greatly simplifies the LPCR assay, but is more expensive.

Preparation of liposome detection reagents

14| Dissolve 58 mg of DOPC, 5.8 mg of rhodamine-DHPE and either 3 mg of monosialoganglioside G_{M1} or 4.2 mg of trisialoganglioside G_{T1b} in chloroform to a final volume of 2–4 ml. The molar ratio of the three components in this solution is 92.2:5.4:2.4. The rhodamine-DHPE is added to determine the concentration of the liposome solution and as a visualization aid during purification. It does not interfere with the real-time PCR measurement.

15| Add the solution to a test tube, and remove the chloroform by incubation in a water bath (heated to 45 °C) under a stream of nitrogen gas.

! CAUTION Chloroform is a carcinogen and can cause liver and kidney damage. It should be handled and disposed of appropriately (see http://www.osha.gov for further information). Evaporation should be carried out under a chemical fume hood.

16| Remove residual chloroform by incubation in a vacuum dryer for at least 4 h.

17| Disperse the dry lipid mixture in 1 ml of 10 mM Tris buffer, pH 7.4, to yield a total lipid concentration of 80 mM. Use a vortex mixer set on high speed and continue until there is no lipid film remaining on the sides or bottom of the tube.

18| Prepare small unilamellar vesicles (SUVs) by sonication with a probe-tip sonicator. Use a sonication program of 10 cycles of 4 min on/1 min off. Immerse the tube in an ice bath throughout the process to minimize sample heating.

19| Centrifuge the resulting SUVs at 1,500*g* in a microcentrifuge for 5 min to remove undispersed lipid and titanium from the probe tip.

20| Combine SUVs (250 μl, 20 μmol total lipid) and reporter (150 μl, 100 μg).

21| To this mixture, add 600 μl of ethanol/calcium chloride solution (8.3 mM $CaCl_2$ in 16.6 mM Tris, pH 7.4, containing 79% (v/v) ethanol). Add the solution dropwise over approximately 30 s with maximum vortex mixing.

▲ CRITICAL STEP The ethanolic/calcium chloride solution must be added slowly to the rapidly vortexed liposome-DNA solution to prevent high local concentrations of calcium, which would lead to undispersed DNA-lipid aggregates.

22| Dialyze the resulting DNA-containing large unilamellar liposomes against 500 volumes of PBS, pH 7.4, for 24 h at 4 °C with two changes of buffer[11].

■ PAUSE POINT The liposome mixture can be stored at 4 °C for up to 1 week.

Purification of the liposome detection reagents

23| Mix the liposome suspension (0.2 ml) with 0.4 ml of 30% (w/v) Ficoll dissolved in PBS, pH 7.4, to give a final concentration of 20% (w/v) Ficoll in PBS. Transfer the liposome suspension to an ultracentrifuge tube in a swinging bucket rotor.

24| Gently layer a 1.2-ml volume of 10% (w/v) Ficoll in PBS, pH 7.4, on top of the liposome suspension. Cover the Ficoll layers with a 0.4-ml layer of PBS, pH 7.4.

25| Centrifuge the discontinuous gradient for 30 min at 100,000*g* at 23 °C.

26| Collect the liposomes at the interface between the saline and 10% (w/v) Ficoll layers. Unencapsulated reporter remains in the lowest Ficoll layer.

27| Dialyze the purified liposomes at 4 °C against 500 volumes of PBS, pH 7.4 (12 h), followed by 500 volumes of 10 mM Tris, pH 7.8 (12 h).

28| Store the purified liposome detection reagent under nitrogen in a sealed dark vial at 4 °C.

■ PAUSE POINT The liposome detection reagent can be stored for up to 6 months with little loss of encapsulated reporter.

For an alternate liposome purification procedure see **Box 1.**

Preparation of blocking liposomes

29| SUVs are used as a blocking reagent in the microtiter plate assay.

BOX 1 | ALTERNATE LIPOSOME PURIFICATION PROCEDURE

An alternate purification procedure is to degrade the unencapsulated reporter with Dnase I and exonuclease III. The DNA-liposomes are then resolved from the free nucleotides by gel permeation chromatography[18].

To the dialyzed liposomes from Step 22, add 2,000 units of pancreatic DNase I, 300 units of exonuclease III and 5 mM $MgCl_2$ to the external aqueous phase.

Incubate the reaction mixture for 3 h at 37 °C, and then stop the reaction by adding 7 mM EDTA.

Remove the nucleotides and enzymes from the DNA-liposomes by elution from a 5 ml Sepharose CL-4B column equilibrated in 10 mM Tris, pH 7.8. Proceed from Step 28.

30| The blocking SUVs are prepared using a lipid mixture of DOPC and rhodamine-DHPE (94.6:5.4), but no ganglioside or reporter is added during the preparation.

31| Prepare and purify the blocking SUVs as described above (Steps 15–19).

32| Store the purified liposome detection reagent under nitrogen in a sealed dark vial at 4 °C.
■ **PAUSE POINT** The blocking liposomes can be stored for up to 3 months.

Characterization of the liposome detection reagents

Determination of total lipid concentration

33| Mix 25–100 µl of liposome solution in a test tube along with 1.5 ml of methanol and 20 µl of 0.1 M NaOH. Vortex the solution and allow it to stand for 5 min.

34| Prepare a blank by substituting PBS for the liposome solution.

35| Read the absorbance of the liposome solution at 560 nm after zeroing the spectrophotometer against the blank.

36| Determine the rhodamine-DHPE concentration using an extinction coefficient of 95,000 $M^{-1}cm^{-1}$ after compensating for the dilution factor.

37| Calculate the total lipid concentration based upon the mole percent of rhodamine-DHPE in the original lipid mixture[8].
▲ **CRITICAL STEP** Normally, the liposome detection reagents require no further characterization beyond the calculation of the total lipid concentration. The effect on the assay of variations in the ganglioside concentration or the number of encapsulated reporters per liposome is compensated for by determining a standard curve using known concentrations of biotoxin. However, a brief discussion of a more complete characterization of the liposome detection reagents is provided for those desiring to develop their own assays using different gangliosides or reporters.

Determination of liposome concentration

38| Determine the hydrodynamic diameter of the liposomes using any dynamic light scattering spectrometer that can measure particle diameters from 10 to 1,000 nm. Follow the manufacturer's instructions for the use of the spectrometer. If a suitable spectrometer is not available, a diameter of 150 nm can be assumed[4].

39| Estimate the number of lipid molecules per liposome (N_{tot}) using equation (1), where d is the hydrodynamic diameter of the liposomes as determined by dynamic light scattering. This equation assumes a bilayer thickness of 4 nm and a lipid headgroup area of 0.71 nm^2 for phosphatidylcholine. The contribution of ganglioside and rhodamine-DHPE to the average headgroup area are ignored in this approximation.

$$N_{tot} = (4.43\text{nm}^{-2}) \times [d^2 + (d - 8\text{nm})^2] \quad (1)$$

40| Estimate the concentration of liposomes in the solution (L_{tot}) using equation (2):

$$L_{tot}(\mu\text{mol ml}^{-1}) = [\text{total lipid}(\mu\text{mol ml}^{-1})]/N_{tot} \quad (2)$$

Determination of reporter concentration

41| Mix 100 µl of liposome solution with 900 µl of 100 mM Octyl-β-D-glucopyranoside. Vortex the mixture and incubate at 37 °C for 15 min. Prepare a corresponding blank using 100 µl of PBS, pH 7.4.

42| Read the optical absorbance of the sample at 260 nm and subtract the corresponding reading for the blank.

43| The total reporter concentration (R_{tot}) is calculated using equation (3):

$$R_{tot}(\mu\text{g ml}^{-1}) = A_{260} \times 0.020(\mu\text{g ml}^{-1}) \times 10 \quad (3)$$

where A_{260} is the optical absorbance of the sample, 0.020 μg ml^{-1} is the absorbance of a 1 μg ml^{-1} solution of β_2-microglobin reporter, and 10 is the dilution factor.

Determination of the number of reporters per liposome

44| Add 1 μl of 1 mM TO-PRO-1 in dimethylsulfoxide to a 1 ml solution of DNA-liposomes diluted 100-fold in PBS, pH 7.4. Prepare a scattering blank by substituting 1 μl of dimethylsulfoxide for the TO PRO-1 solution[11].

45| Measure the fluorescence emission of the liposome solution at 531 nm using an excitation wavelength of 514 nm and 5 nm slit widths. Subtract the fluorescence of the blank solution from that of the sample.

46| Add 20 μl of 100 mM Triton X-100 to both liposome solutions, vortex, and allow the solutions to incubate in capped tubes at 37 °C for 15 min. This serves to rupture the liposomes.

47| Re-measure the fluorescence emission of the liposome solution at 531 nm using an excitation wavelength of 514 nm and 5 nm slit widths. Subtract the fluorescence of the blank solution from that of the sample and correct for the dilution of the detergent solution.

48| Determine the percent encapsulation as the ratio of fluorescent intensity before to that after the addition of 20 μl of 100 mM Triton X-100 to rupture the liposome detection reagents.

49| Determine the concentration of encapsulated reporter by using the percent encapsulation and the total reporter concentration determined in Step 43.

50| Determine the number of reporters per liposome by dividing the concentration of encapsulated reporter by the liposome concentration (L_{tot}) determined in Step 40.

LPCR microtiter plate assay for CTBS

51| Coat each well of a 96-well EIA high-binding flat plate with 150 μl of anti-CTBS mouse monoclonal antibody (1.0 μg ml^{-1}) in coating buffer.

52| Cover the microtiter plate with a plate sealer and incubate the plate at 4 °C on a plate shaker at 600 r.p.m. for 18 h.

53| Aspirate the coating buffer and wash the plate wells five times with wash buffer A using a microtiter plate washer.

54| Add 300 μl of blocking buffer to each well and incubate the plate for 2 h at 23 °C.

55| Aspirate the blocking buffer and wash the wells twice with wash buffer A.

56| Add 150 μl of serially diluted CTBS (concentration range: 10^{-14} to 10^{-19} M in dilution buffer) or 150 μl of dilution buffer (blank) to the plate wells. Also, include a 'no template' control. Prepare 3–5 replicates for each antigen concentration, including the blank and control. Incubate the plate at 23 °C for 1 h.

57| Aspirate the sample solutions and wash the wells five times with wash buffer A and twice with wash buffer B.

58| Add 150 μl of blocking liposomes (2.0 μmol ml^{-1} total lipid, diluted 1:1,000 in dilution buffer) to the plate wells, and incubate the plate at 23 °C for 1 h.

59| Aspirate the blocking liposome solution and wash the plate three times with wash buffer B.

60| Add 150 μl of the monosialoganglioside G_{M1}-containing liposome detection reagent (0.8 μmol ml^{-1} total lipid, diluted 1:1,000 in dilution buffer) to the wells, and incubate the microtiter plate at 23 °C for 1 h.

61| Aspirate the detection liposomes and wash the wells ten times with wash buffer B.

62| Degrade any unencapsulated DNA by adding 150 IU of pancreatic DNase I in 100 μl of digestion buffer to each plate well. Cover with a plate sealer, and incubate the plate at 37 °C on a plate shaker with gentle shaking for 30 min (ref. 12).

63| Heat the plate at 80 °C for 10 min to inactivate the DNase I. Aspirate the enzyme solution and wash the wells five times with wash buffer B.

64| Add 100 µl of lysis buffer to the wells, cover with a plate sealer and incubate the plate on a shaker at 600 r.p.m. for 15 min at 23 °C. The lysis buffer serves to rupture the membranes of the liposomes, which releases the encapsulated reporters. Blocking with a nonspecific DNA to prevent loss of reporter is not required.

▲ **CRITICAL STEP** All of the above steps are critical to the success of the LPCR assay. A high concentration of DNase I is used since DNA absorbed to the plate walls or the outer surface of the liposomes can be difficult to digest. It is acceptable to use a partially purified grade of DNase I (non RNase free) to minimize cost.

■ **PAUSE POINT** The plate can remain sealed overnight prior to analysis by real-time PCR.

? TROUBLESHOOTING

LPCR microtiter plate assay for BoNT/A

65| The LPCR microtiter plate assay for BoNT/A is carried out as described above with the following modifications.

- Trisialoganglioside G_{T1b}-containing liposomes are used as the detection reagent due to the high affinity of this ganglioside for BoNT/A[13].
- Corning flat-bottom EasyWash high-binding 96-well plates are used.
- The capture antibody (anti-BoNT/A) concentration in coating buffer is 2.5 µg ml^{-1}.

Real-time PCR

66| Add a 2-µl aliquot from each microtiter plate well to a 50-µl PCR reaction mixture prepared from Taqman universal PCR Mastermix, which contains Taqman buffer A; 3.5 mM $MgCl_2$; 200 µM each of dATP, dCTP and dGTP; 400 µM dUTP; 1.25 units of AmpliTaq Gold; and 0.5 units of AmpErase UNG.

67| Add forward and reverse primers (300 nM each) along with the probe (200 nM).

68| Set up and initiate a PCR protocol consisting of a 2-min UNG incubation step at 50 °C and a 10-min AmpliTaq Gold activation step at 95 °C. Then perform 40 cycles of PCR, where each cycle consists of a 15-s denaturation step at 95 °C and a 1-min annealing/extension step at 60 °C.

69| The forward (β_2M-246F) and reverse (β_2M-330R) primer sequences were given in Step 5. The fluorescent probe is:

5′-[VIC] TGA TGC TGC TTA CAT GTC TCG ATC CCA[TAMRA]-3′

Data analysis

70| For each antigen concentration in the dilution series, including the blank, calculate the mean C_t value and the standard deviation of the 3–5 replicate measurements from the real-time PCR analysis. The no template control should have a C_t value of 37 to 40. A lower value could indicate the presence of reporter contamination in the PCR reaction mixture[14].

71| Construct a standard curve by plotting the average C_t values and their standard deviations for the serially diluted antigen versus the $\log_{10}$ of the antigen concentration. Perform a linear regression analysis of the data to obtain the equation for the standard curve and the 95% confidence limits. The linear correlation coefficient should be ≥ 0.98. Data analysis is performed with Origin version 7.0 or equivalent software.

72| Determine the detection threshold of the LPCR assay, which is defined as the average C_t value of the blank minus three times the standard deviation of the blank, as is used for immuno-PCR assays[5,15].

73| Convert the C_t value of an unknown sample into toxin concentration by interpolation using the standard curve and linear regression equation determined with the serially diluted standards. The unknown sample must be within the dynamic range of the standard curve, which is typically 5–6 orders of magnitude.

▲ **CRITICAL STEP** Always prepare the standard curve using the same sample matrix as the unknowns. For example, if the samples to be analyzed are urine specimens, the standards should also be prepared in urine. Always include serially diluted standards with every plate to ensure the highest possible precision in the LPCR measurements. The PCR amplification efficiency[16] (E) can normally be determined from the slope of the standard curve as $E = 10^{-1/\text{slope}}$. If the efficiency of a PCR reaction is 100%, a $\log_{10}$ increase in reporter concentration will require about 3.3 cycles, which yields a value of 2 for E. For the LPCR assays reported here, the slopes are ~ 1, thus $E > 2$ indicating efficiencies greater than 100%. This arises from the fact that the slopes of the LPCR assays are a function of the change in biotoxin concentration superimposed upon a relatively constant level of nonspecific binding of the DNA-liposomes. For this reason, the apparent amplification efficiencies are greater than 100%, a fact that does not in itself compromise the accuracy of the assay[16].

● TIMING

Steps 1–13: 2 d (if amplifying existing β_2-microglobin transcript), or 2–4 weeks (if cloning is required)
Steps 14–22: 2 d
Steps 23–28: 1 d
Steps 29–32: 6 h
Steps 33–50: 2 h
Steps 51–65: 4 h
Steps 66–73: 2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reasons	Solutions
Assay background high		
Steps 54, 56, 58, 60	Poor quality BSA	Use RIA-grade BSA
Steps 29–32, 58	Poor SUV blocking	Use fresh blocking liposomes or a higher SUV concentration
Step 60	Detection reagent concentration too high	Use a higher dilution of DNA-liposomes
Steps 62–63	Incomplete DNA digestion	Use fresh DNase I, a higher enzyme concentration, or a longer digestion time
Steps 51–64	Improper reagents or assay conditions	Check buffer compositions, check proper operation of plate washer, increase number of wash cycles
Steps 66–69	Contaminated PCR reaction mixture	Check no-template control, if C_t below 37 replace PCR reagents
Loss of assay sensitivity		
Steps 14–28	Detection reagent too old	Prepare fresh DNA-liposomes
Step 60	Detection reagent concentration too low	Use a lower dilution of DNA-liposomes
Step 60	Non-optimal pH	Use pH 7.8 for detection reagent binding
Step 51	Capture antibody too old or too dilute	Use fresh capture antibody or decrease capture antibody dilution
Steps 14–28	Too little ganglioside in detection reagent	Prepare fresh DNA-liposomes using proper lipid composition
Poor dynamic range		
Step 60	Formation of confluent liposome monolayer[8]	Use a lower concentration of detection reagent
Steps 66–69	Poor PCR reaction conditions	Check real-time PCR instrument and protocol. Replace PCR reagents
Poor reproducibility		
Step 54	Too few replicate measurements	Increase the number of replicates per sample. Particularly important for low toxin concentrations[4]
Steps 49–67	Poor technique	Check for proper operation of pipetters, plate washer, etc.

ANTICIPATED RESULTS

For biotoxin assays performed in deionized water, detection thresholds down to 10–50 molecules in 150 µl of water are typical (zeptomolar (10^{-21}M) to attomolar (10^{-18}M) concentration range). The lower concentration limit is determined predominately by the binding affinities of the capture antibody and ganglioside for the biotoxin. The affinity of gangliosides for biotoxins can vary widely[1,9]. For example, the detection threshold for an assay for tetanus toxin in deionized water using G_{T1b}-containing liposomes is ~325 molecules (unpublished data) due to the lower binding affinity of G_{T1b} for tetanus toxin relative to BoNT/A.

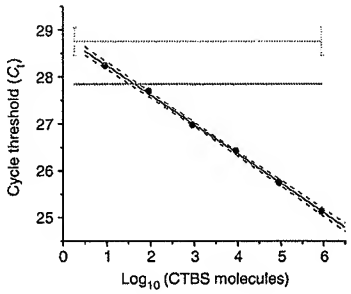

Figure 2 | Results of an LPCR assay of human urine spiked with CTBS. A urine specimen was collected from a healthy human male volunteer. The urine specimen was spiked with cholera toxin beta subunit (CTBS), filtered through a 0.2-µm polycarbonate filter to remove any particulates, and the pH of the specimen was adjusted to 7.8 using 0.1 M NaOH. Four replicate liposome polymerase chain reaction (LPCR) measurements were carried out for each of six serial dilutions of CTBS in the urine specimen (concentration range: 10^{-14} to 10^{-19} M) plus an unspiked urine blank. A plot of the average serial dilution cycle threshold (C_t) values versus the log of the number of CTBS molecules per plate well for the four replicate measurements is shown. The solid black circles are the average serial dilution C_t values. The solid red line is a linear regression fit to the C_t values, and the dashed blue lines are the 95% confidence limits. The solid horizontal orange line denotes the average blank C_t value. The standard deviation of the blank is drawn at each end of this line. The solid horizontal green line that intersects the linear regression line indicates the detection threshold of the assay. The detection threshold for this LPCR assay is 43 ± 10 molecules of CTBS (0.5 attomolar or 0.09 fg ml^{-1}). The assay dynamic range is almost five orders of magnitude. The slope of the linear regression fit of the data is −1.02 ($r^2 = 0.998$).

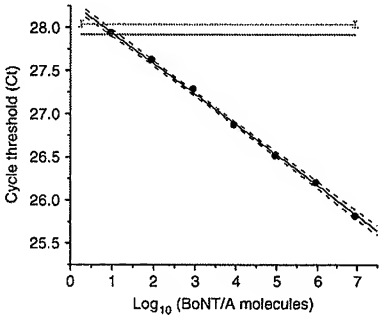

Figure 3 | Results of an LPCR assay of deionized water spiked with BoNT/A. Deionized water (18 MΩ) was spiked with botulinum neurotoxin type A (BoNT/A), filtered through a 0.2-μm polycarbonate filter to remove any particulates, and the pH of the specimen was adjusted to 7.8 using 0.1 M NaOH. Four replicate LPCR measurements were carried out for each of six serial dilutions of BoNT/A (concentration range: 10^{-14} to 10^{-19} M) plus an un-spiked water blank. A plot of the average serial dilution cycle threshold (C_t) values versus the log of the number of BoNT/A molecules per plate well for the four replicate measurements is shown. The symbols are as defined in **Figure 2**. The detection threshold is 12 ± 4 molecules of BoNT/A (0.1 attomolar or 0.02 fg ml^{-1}). The assay is linear over approximately five orders of magnitude. The slope of the linear regression fit of the data is −0.632 ($r^2 = 0.998$).

The selection of the capture antibody is critical, as it cannot compete with the ganglioside for the same epitope. For example, attempts to create and LPCR assay for ricin using the monoclonal antibody 2R1 (clone CP23)[17] were unsuccessful (unpublished data) as both apparently compete for the same epitope on the ricin A-chain. In general, polyclonal antibodies are more effective than monoclonal antibodies for use as the capture antibody in the LPCR assays. Biotoxin assays using environmental or biological specimens have higher detection thresholds due to the higher background (DNA-liposome non-specific binding) resulting from the more complex matrix. Detection thresholds of 50–500 molecules in 150 μl of solution [attomolar (10^{-18}M) concentration range] are typical for these more complex specimens.

Representative results obtained for LPCR assays of CTBS and BoNT/A are the following[4] (**Figs. 2** and **3**):

- CTBS in deionized water. The detection threshold is 10 ± 3 molecules of CTBS [17 yoctomoles (17×10^{-24}) derived from a 113-zeptomolar solution (0.02 fg ml^{-1})] based upon the linear regression and 95% confidence limits derived from the sample data. The dynamic range of the assay is almost five orders of magnitude.
- CTBS in human urine. The detection threshold is 43 ± 10 molecules of CTBS (71 yoctomoles derived from a 0.5-attomolar solution (0.09 fg ml^{-1})). The dynamic range of the assay is almost six orders of magnitude.
- CTBS in farm runoff water. The detection threshold is 377 ± 168 molecules of CTBS [0.6 zeptomoles derived from a 4-attomolar solution (0.75 fg ml^{-1})]. The dynamic range of the assay is almost five orders of magnitude.
- BoNT/A in deionized water. The detection threshold is 12 ± 4 molecules [20 yoctomoles derived from a 0.1 attomolar solution (0.02 fg ml^{-1})]. The assay is linear over approximately five orders of magnitude.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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A liposome-PCR assay for the ultrasensitive detection of biological toxins

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We describe an ultrasensitive immunoassay for detecting biotoxins that uses liposomes with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as a detection reagent. After immobilization of the target biotoxin by a capture antibody and co-binding of the detection reagent, the liposomes are ruptured to release the reporters, which are quantified by real-time PCR. Assays for cholera and botulinum toxins are several orders of magnitude more sensitive than current detection methods.

The potential use of biological toxins as weapons of mass destruction has created an urgent need for rapid and highly sensitive assays for their detection. We describe one such assay method, liposome polymerase chain reaction (LPCR), which is robust and straightforward to perform, yet uses inexpensive and stable reagents.

We present an overview of the LPCR method with an assay for the detection of cholera toxin beta subunit (CTBS) in deionized water. Detailed procedures for this assay and one for botulinum neurotoxin type A (BoNT/A) are provided in the **Supplementary Methods** online. About 60 copies of an ~80-bp dsDNA segment (the reporter) are encapsulated inside a single-shell liposome. The reporter serves as a PCR amplification substrate for quantification of CTBS. Approximately 2,500 molecules of monosialoganglioside G_{M1} are incorporated into the bilayer of the liposome to serve as a nonspecific receptor for CTBS[1]. A cross-section of the resulting liposome detection reagent is represented in **Figure 1**.

The LPCR assay follows the familiar sandwich enzyme-linked immunosorbent assay (ELISA) format. A monoclonal antibody against CTBS is adsorbed inside the wells of a microtiter plate and serves to provide specificity by capturing CTBS from the sample solution. Nonspecific protein binding is blocked using bovine serum albumin. Each well then receives 150 µl of serially diluted CTBS (a concentration range of 10^{-14} to 10^{-19} M) or buffer (blank). Nonspecific liposome binding is blocked by the addition of small unilamellar vesicles composed of phosphatidylcholine. The liposome detection reagent is added, and the plate is incubated at 23 °C for 1 h. The plate wells are then rinsed several times with PBS. Unencapsulated DNA is degraded by the addition of pancreatic DNase I solution, followed by incubation at 37 °C for 30 min. The DNase I is then inactivated by heating the plate to 80 °C for 10 min, and the encapsulated reporters are released by rupturing the liposomes with Triton X-100. An aliquot from each microtiter plate well is added to a PCR reaction mixture, and the samples are analyzed by real-time PCR (**Supplementary Fig. 1** online).

Figure 2a shows the average cycle threshold (Ct) values obtained by real-time PCR versus the log of the number of CTBS molecules per plate well for four replicate LPCR measurements of CTBS in deionized water. The detection threshold of the assay is defined as the average Ct value of the blank minus three times the standard deviation of the

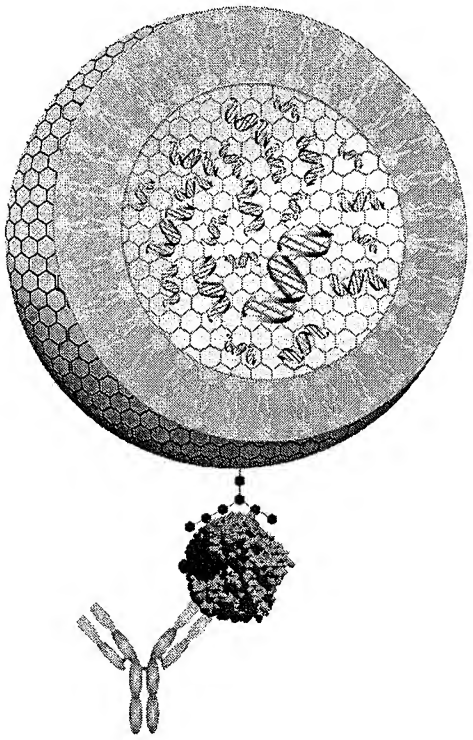

Figure 1 Representation of a liposome detection reagent in cross section. The dsDNA reporters (green with red bars) are encapsulated inside the bilayer (yellow) into which a monosialoganglioside G_{M1} receptor (blue) has been incorporated. The liposome is shown bound to a CTBS pentamer, which is co-bound to a capture antibody.

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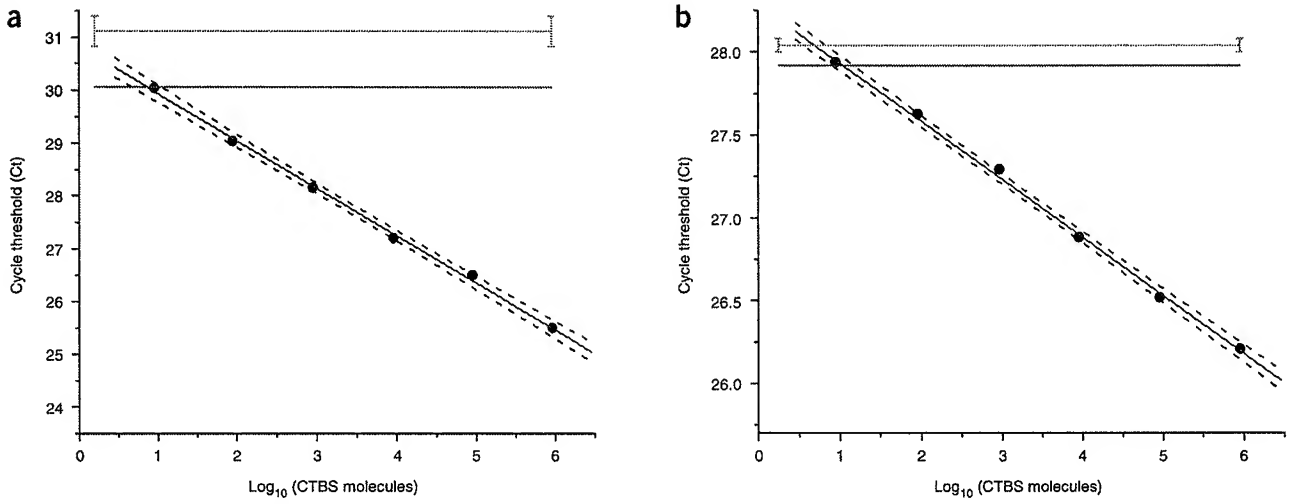

Figure 2 Plots of the average serial dilution Ct values versus the log of the number of molecules per plate well for the four replicate measurements of **(a)** CTBS in deionized water and **(b)** BoNT/A in deionized water. The concentration ranges for both CTBS and BoNT/A were 10^{-14} to 10^{-19} M. Solid black circles are average Ct values. The solid red line is a linear regression fit ($r^2 = 0.999$) to the average Ct values, and the dashed blue lines are the upper and lower 95% confidence limits. The solid horizontal orange line denotes the average blank Ct value, with the standard deviation of the blank drawn at each end of this line. The solid horizontal green line intersecting the linear regression line indicates the detection threshold of the LPCR assay as defined in the text.

blank, as is used for immuno-PCR assays[2]. The detection threshold for this assay is 10 ± 3 molecules of CTBS (0.02 fg/ml) based upon the linear regression and 95% confidence limits derived from the sample data. The dynamic range of the assay is almost five orders of magnitude. The LPCR dose-response curve is similar in linearity and dynamic range to those seen with conventional immuno-PCR[2,3], but with higher sensitivity. The specificity of the assay for CTBS was demonstrated in an LPCR assay in which tetanus toxoid was substituted for CTBS; this assay revealed no detectable reporter amplification above background levels (not shown).

LPCR assays were also performed on a specimen of field run-off water collected from a local farm and a specimen of human urine, both spiked with CTBS (**Supplementary Figs. 2** and **3** online). The detection threshold for the water specimen is 377 ± 168 molecules of CTBS (0.75 fg/ml). The detection threshold for the urine specimen is 43 ± 10 molecules of CTBS (0.09 fg/ml).

To detect BoNT/A in deionized water, we used a commercially available affinity-purified polyclonal rabbit IgG antibody against this biotoxin. The liposome detection reagent was prepared as described above, but with 2 mol% trisialoganglioside G_{T1b} in place of monosialoganglioside G_{M1}. Trisialoganglioside G_{T1b} is a component of the binding site for the carboxy-terminal half of the 100-kDa heavy chain of BoNT/A[4]. Other minor changes relative to the assay for CTBS are described in the **Supplementary Methods**. The results of this assay are shown in **Figure 2b**. The detection threshold is 12 ± 4 molecules (0.02 fg/ml). The assay is linear over approximately five orders of magnitude.

The sensitivities of the LPCR assays for CTBS and BoNT/A are compared with those of other biotoxin assay methods in **Supplementary Table 1** online. The LPCR detection thresholds for CTBS and BoNT/A are 2–3 orders of magnitude lower than those reported by the most sensitive assays currently in use, while maintaining high specificity and having assay times equal to or shorter than those of most biotoxin assays.

LPCR offers several advantages over current biotoxin detection methods. First, derivatization of the reporter or ganglioside receptors is not required. The reporter is freely encapsulated inside the liposomes, and the ganglioside receptors spontaneously partition into the bilayer as the liposomes are formed. This greatly simplifies the preparation and purification of the detection reagent. Second, the use of real-time PCR, rather than end point PCR, improves the quantitative accuracy of the assay; it also allows for improved precision by performing replicate measurements on the samples and applying statistical treatment to the data[5]. Third, LPCR displays 100–1,000 times greater sensitivity than previous assays for biological toxins (see **Supplementary Table 1**). This is due, in part, to the high number of reporters per binding event and the low nonspecific binding of the liposome detection reagents. Fourth, sequestration of the reporters inside the liposomes offers two distinct advantages not possible with other immuno-PCR-based assays. The reporters are protected from chemical or enzymatic degradation by impurities present in the sample that are incompletely removed during the wash steps. This substantially reduces the possibility of false-negative results. The more important advantage of encapsulating the reporters is that DNase I can be used to degrade any contaminating DNA present in the microtiter plate wells immediately before the rupture of the liposomes by detergent. Thus, DNA contamination from the assay environment, from incomplete purification of the liposome detection reagents, from carryover by pipette tips or plate washer nozzles, or genomic DNA contamination remaining from the samples can all be eliminated. This substantially reduces the possibility of false-positive results and improves the sensitivity and precision of the assay.

The application of LPCR could be greatly expanded by coupling antibodies to the liposome surface as receptors in place of gangliosides. Phospholipid anchors for antibodies are commercially available, and they will spontaneously partition into the bilayer. A number of straightforward coupling chemistries exist for linking whole antibodies, or Fab′ fragments, to the phospholipid anchors[6]. In addition, the

encapsulated dsDNA reporter can be sequence coded to the antibody covalently linked to the liposome detection reagent, which would allow simultaneous detection of multiple antigens. We are currently using this approach for the detection of additional chemical and biological warfare agents and for the detection of biomarkers for cancer and other diseases.

Note: Supplementary information is available on the Nature Biotechnology website.

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RELATED PROCEEDINGS APPENDIX

NONE